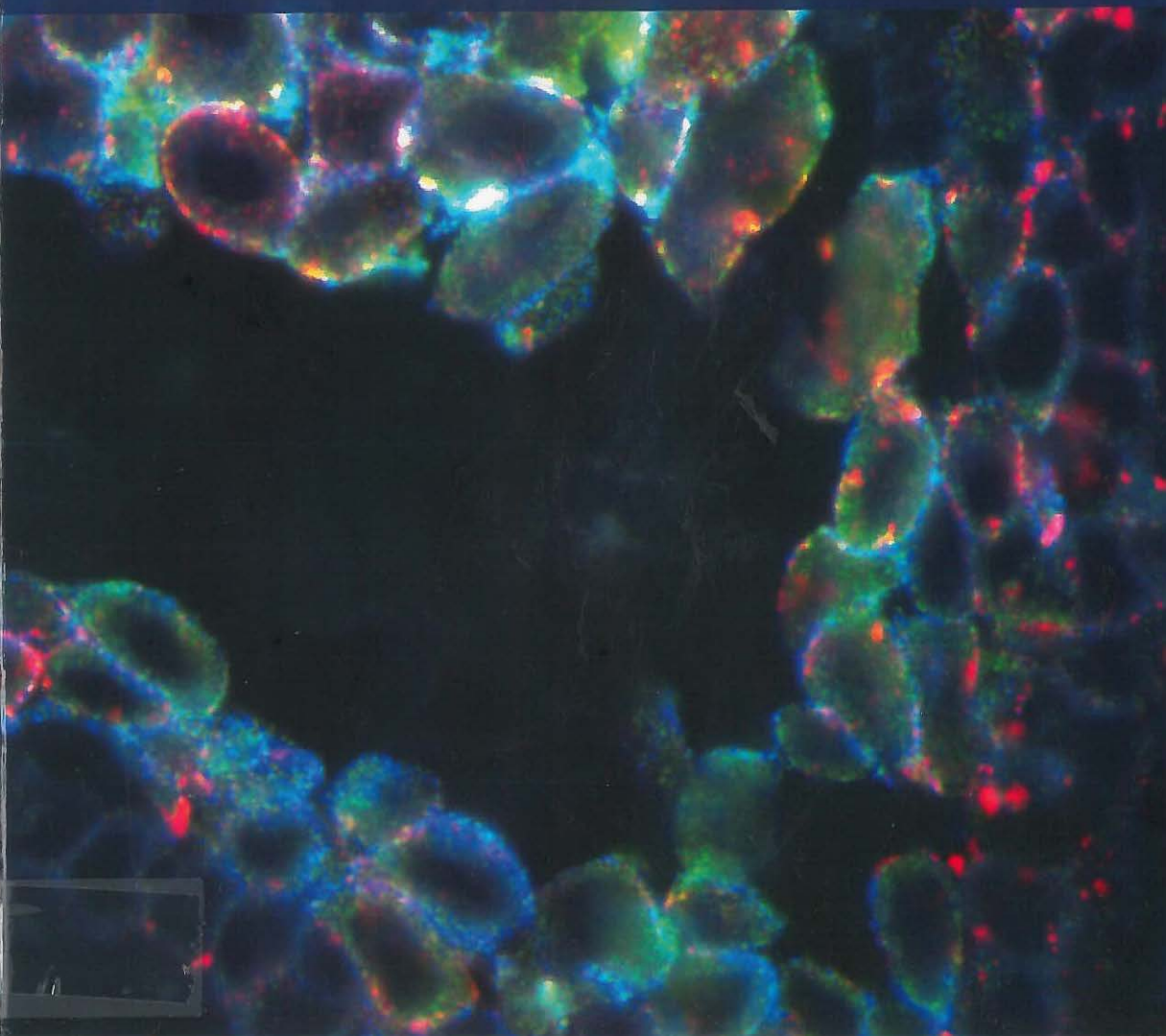
Pemphigus pathogenesis : insight from patient skin studies

Oktarina, Dyah Ayu Mira

D.A.M. Oktarina

Pemphigus pathogenesis

Insights from patient skin studies



Pemphigus pathogenesis

Insights from patient skin studies

D.A.M. Oktarina

1. The IgG induced rearrangement of desmogleins 1 and 3 is responsible for the granular IgG deposition pattern in pemphigus patient skin *(in this thesis)*.

2. The aggregation of desmoglein is specific for pemphigus (*in this thesis*).

3. Desmoglein 1 aggregation but not desmoglein 3 aggregation correlates with non-acantholytic intercellular widening (*in this thesis*).

4. Cadherin molecules that have IgG bound to it are not properly incorporated in desmosomes what leads to severe desmosome assembly problems (*in this thesis*).

5. The shed desmoglein 1 ectodomain in the basal membrane zone of pemphigus erythematosus skin indicates the presence of a to pemphigus foliaceus additional pathogenic mechanism (*in this thesis*).

6. The desmoglein aggregates in pemphigus foliaceus skin are cleared from the membrane by endocytosis (*in this thesis*).

7. Observations from experimental models claimed to be relevant for pathogenesis should be verified in patient biopsies (*in this thesis*).

8. Doing a PhD is like a long-term relationship. There are good times and some dreadful ones; it is a big help when you like what you have chosen (*Joan Bolker Ed.D in Writing Your Dissertation in Fifteen Minutes a Day*).

9. He who knows best knows how little he knows (*Thomas Jefferson*).

10. The knowledge of the paddy or rice plant: the bigger the grain grows, the lower the plant bends. As one gains more knowledge, one becomes more humble (*Indonesian proverb*).

senger of Allah (peace be upon him) said, "For those who go on a quest for knowledge, Allah will smooth the path to Paradise" (*Hadith narrated by Ibn Abbas*).

Financial support for the publication of this thesis was provided by:
ABBOTT B.V.; Galderma; Graduate School of Medicine Groningen (GUIDE); Schlumberger Foundation; University of Groningen (Rijksuniversiteit Groningen).

Printed by: Proefschriftmaken.nl || Printyourthesis.com
Published by: Uitgeverij BOXPress, Oisterwijk

ISBN: 978-90-8891-210-8

rijksuniversiteit groningen

Pemphigus pathogenesis

Insights from patient skin studies

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 8 december 2010
om 11.00 uur

door

Dyah Ayu Mira Oktarina

geboren op 27 oktober 1980
te Yogyakarta, Indonesië

Promotor:	Prof. dr. M.F. Jonkman
Copromotor:	Dr. H.H. Pas
Beoordelingscommissie:	Prof. dr. H. Soebono
	Prof. dr. A. Vissink
	Prof. dr. H. van Goor

Table of Contents

List of abbreviations

PV:	pemphigus vulgaris
PF:	pemphigus foliaceus
IgG:	immunoglobulin G
ICS:	intercellular substance
Dsg:	desmoglein
Dsc:	desmocollin
DP:	desmoplakin
PG:	plakoglobin
EPK:	envoplakin
E-cad:	E-cadherin
B-cat:	β-catenin
PKP3:	plakophilin 3
EEA:	early endosomal antigen
PLEC:	plectin
ELISA:	Enzyme-Linked ImmunoSorbent Assay
BMZ:	basement membrane zone
ET:	exfoliative toxin
ETA:	exfoliative toxin A
ETB:	exfoliative toxin B
SSSS:	staphylococcal scalded skin syndrome

Chapter 1

Introduction

D.A.M. Oktarina

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

The autoimmune disease pemphigus

The term "pemphigus" is originally derived from the ancient Greek word, pemphix, meaning bubble or blister. Pemphigus refers to a group of chronic life-threatening autoimmune disorders, which is characterized clinically by exfoliation or blistering of the skin and/or blistering of the mucous membranes, and histologically by cleft formation in the epidermis and/or epithelium. The basic pathology for blister formation is the loss of cell-cell adhesion, called acantholysis, which is caused by circulating autoantibodies that are directed to the cell surface of keratinocytes.

Based on the level of the intraepidermal split formation, pemphigus is classified in two main forms: pemphigus vulgaris (PV) and pemphigus foliaceus (PF). In PV the blisters are located just above the basal and suprabasal layers of the epidermis, while in PF the blisters are formed within the upper layers. Other forms of pemphigus are paraneoplastic pemphigus (PNP) that is related with neoplasm, commonly lymphoma, and drug induced pemphigus that develops after the administration of drugs such as penicillamine and captopril.

Epidemiology of pemphigus

Pemphigus is the most common cause of mortality among the dermatological diseases. The incidence of pemphigus ranges from 0.76 until 5 cases per million per year. However, the prevalence and incidence is higher in populations of Jewish ancestry. In several countries like Israel, Iran, and Tunisia, this disease is more endemic with an incidence of 1.6-5 cases per 100.000 [1,2]. The occurrence of this disease is primarily between the third and fifth decades without sex preference [3].

Diagnosis of pemphigus

The diagnosis of pemphigus is based on the following major criteria: clinical, histopathological, and immunofluorescence findings [4]. PV evolves with flaccid blisters and erosions in mucosae of cornea, oral and nasal cavity, laryngo-pharynx, esophagus, anal canal, vagina, and the internal portion of the lips with non-keratinized stratified squamous epithelium (Figure 1b), and in approximately half of the cases also in the skin (Figure 1a). Histopathological examination shows suprabasal acantholysis in the epidermis, leaving loosened basal keratinocytes that remain attached to the dermoepidermal basement membrane standing out like a row of "tombstones" (Figure 2a and 2b). In contrast, PF forms crusted and scaly lesions only in the skin (Figure 1c and 1d) due to superficial acantholysis in the subcorneal or granular layer of the epidermis (Figure 2c).

By indirect immunofluorescence microscopy on monkey oesophagus, guinea pig lip, or human skin pemphigus sera demonstrate the typical intercellular substance (ICS) staining, which is also referred to as "chicken wire" or "honeycomb" pattern. Direct immunofluorescence microscopy of the perilesional and uninvolved skin and/or mucosa reveals tissue-bound IgG and/or IgA. The immunoglobulin deposition is commonly accompanied by the addi-

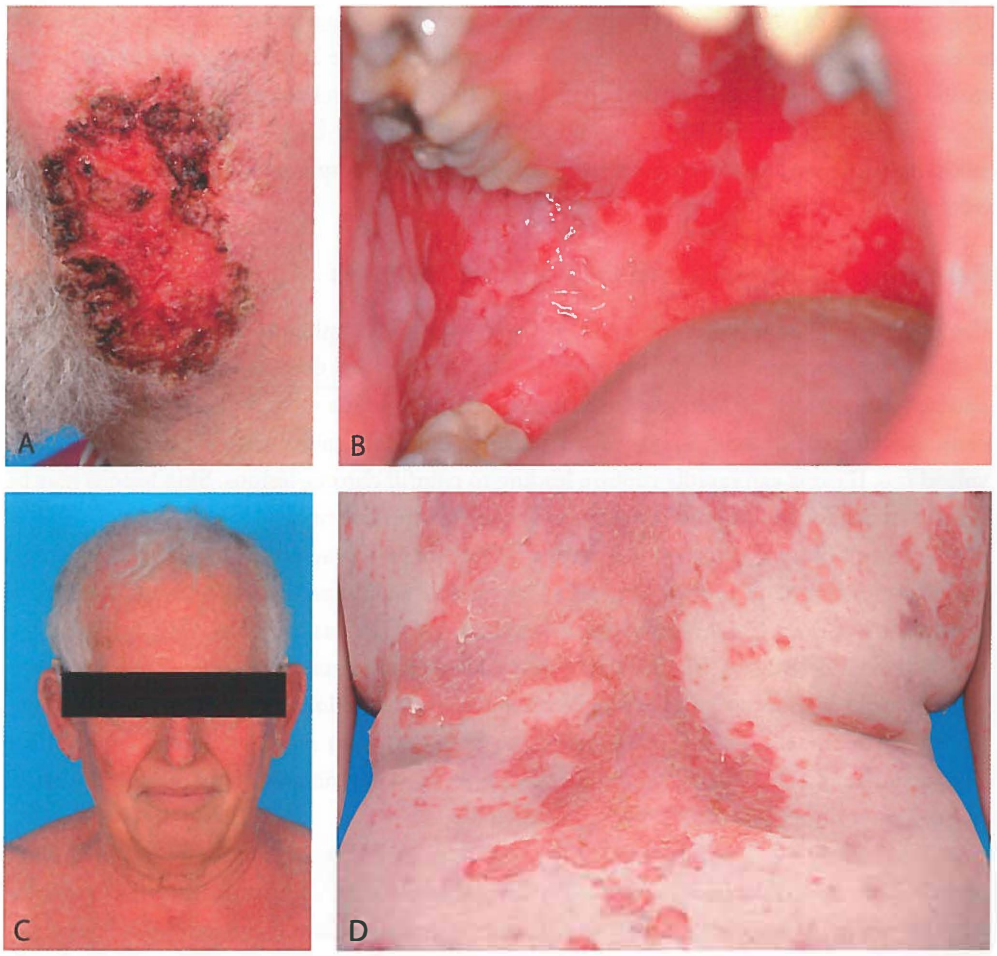


Figure 1. Clinical features of pemphigus vulgaris and pemphigus foliaceus. **(A)** Flaccid blisters and cutaneous erosion in the skin. **(B)** Painful erosion in the oral mucosa of pemphigus vulgaris patient. **(C)** Crusted and scaly lesion on the face and seborrhoic areas. **(D)** Crusted lesions on the back of a pemphigus foliaceus patient.

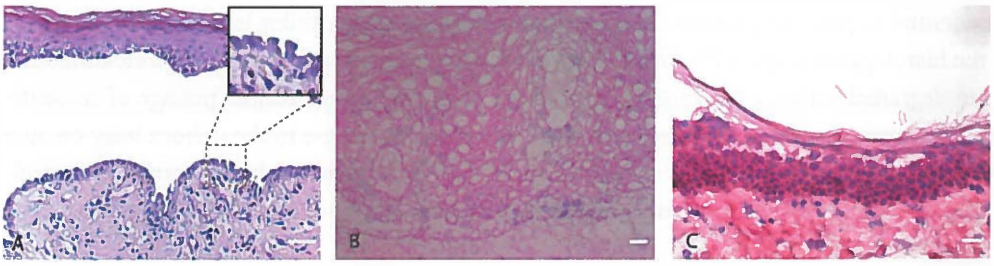


Figure 2. Histology of pemphigus vulgaris and pemphigus foliaceus. **(A)** acantholytic split above the first (basal) and second (suprabasal) epidermal cell layers in the skin, tombstone (inset) **(B)** and above the first (basal) layers in the mucosa of patient with pemphigus vulgaris. **(C)** Subcorneal acantholysis in the skin of a pemphigus foliaceus patient. White bar is 20 micrometers.

tional presence of complement C3. Immunoblot and ELISA analysis of pemphigus sera assist in diagnosing pemphigus. ELISA kits for the analysis of the autoantibody response to the major target antigens desmoglein (Dsg) 1 and 3 are commercially available. This allows for discrimination of PV and PF and moreover the titres of anti-Dsg1 and to a lesser extent of anti-Dsg3 IgG correlate with the disease activity [5]. The desmogleins will be discussed further below.

Pemphigus subtypes and their target antigens

Pemphigus vulgaris (PV) is the most common type of pemphigus. PV is characterized by flaccid blisters or erosion of the mucous membranes (mucosal dominant pemphigus vulgaris, and sometimes also of the skin (mucocutaneous pemphigus vulgaris). The oral mucosa is primarily affected in the majority of the patients, but other mucous membranes may be involved too. Blisters can rapidly rupture leading to painful chronic lesions. Skin lesions may be formed at any location of the integument but mostly in the trunk.

Pemphigus vegetans is a variant of PV which is characterized by cutaneous blisters and pustules that tend to form into verruciform and papillomatous vegetations. The typical distribution of these lesions is in the intertriginous area, such as the axilla. Oral lesion similar to PV is commonly present. There are two ends of a spectrum: the severe Neumann type and the mild Hallopeau type. In the Neumann-type vegetations or papillomatous granulations with peripheral pustules develop on the denuded areas around orificiae, while in the Hallopeau-type pustules that are rapidly followed by vegetations in intertriginous areas are more commonly seen.

Pemphigus herpetiformis is a clinical variant of PV, as autoantibodies against Dsg1 and Dsg3 are demonstrated in pemphigus herpetiformis sera [5]. This pruritic type of pemphigus shows skin lesion resembling those of dermatitis herpetiformis, eosinophillic spongiosis without obvious acantholysis as well as by the presence of circulating and tissue-bound antibodies against the keratinocyte surface [6].

Neonatal pemphigus is a subtype of PV. This type of pemphigus can occur because of the transfer of anti-Dsg3 IgG4 from the mother to the unborn child as the IgG4 has the potential to pass the placenta. After birth, the newborn may exhibit lesions in the skin with the histology findings of PV. These erosions will disappear after the circulating autoantibodies are degraded within a few months. In the case of PF, the transplacental passage of autoantibodies from the affected mother does not cause the skin disease to the unborn baby because the subcorneal layer in infants contains Dsg3 as well as Dsg1 where both contribute intercellular strength in the desmosome, while in the subcorneal layer of adults' skin the desmosomes contain only Dsg1 [7].

A rare variant of PV is that of a localized type where skin lesions, mostly at the nose, develop in the absence of mucosal lesions [8]. These patients have the typical suprabasal PV split and antibodies to Dsg3. The pathomechanism of this rare type is not completely understood and is in complete contrast with the current concepts of pemphigus acantholysis that will be

discussed further below.

Pemphigus foliaceus affects the face, scalp, chest and upper back with scaly crusted lesion with erythema. In PF the blister forms in the superficial layers of the epidermis, i.e., the subcorneal or granular layer. In contrast to PV, there is no involvement of mucous membranes. In central and southern Brazil, there is an endemic form of pemphigus foliaceus, which is called "fogo salvagem" meaning wildfire. This endemic form affects children and young adults. The association with insect bites suggests that this is a transmissible disease with acquired immunity in adulthood. Another well-studied endemic area is located in Tunisia while so far undocumented areas seem to exist in Tanzania and Tibet [9].

Figure 3. The clinical picture of pemphigus erythematosus shows scaly crusted lesions on the face, particularly in the malar region, nose bridge and seborrhoic areas.

Pemphigus erythematosus is considered a localized variant of PF. This subtype has a distinct clinical feature from PF. This localized form of PF shares some clinical pictures with lupus erythematosus as its distribution involves the malar region of the face and the seborrhoic areas (Figure 3). Immunopathologically, this form differs from PF since IgG and C3c are deposited in the epidermal basement membrane zone as well as on cell surfaces of keratinocytes. This finding interested me to observe whether this particular deposition pattern might reveal an additional pathogenic mechanism. (Chapter 4. Desmoglein 1 ectodomain is present in basement membrane zone deposits of IgG and complement in pemphigus erythematosus)

Paraneoplastic pemphigus (PNP) is a distinct form of pemphigus which is characterized by a painful and severe oral and conjuctival erosion and lesion of the palm and soles. Lymphoma and other haematological disorders are related and precede this disease [10]. Besides Dsg1 and 3, several additional target antigens have been identified, including plakin family proteins such as desmoplakin, envoplakin, and periplakin [11-14], bullous pemphigoid antigen 1 (BP230) [10], a yet unidentified 170-kDa molecule [15], and plectin [16].

Drug-induced pemphigus is commonly induced by drugs that contain thiol or sulfur that can be converted to thiols, such as D-penicillamine, captopril, propanolol, indometacin, phenylbutazon, pyritinol, and piroxicam. Clinically, the picture of this disease may resemble either PV or PF. Oral lesion is rare. Immunofluorescence finding is positive with pemphigus pattern. Autoantibodies to both Dsg1 and Dsg3 are commonly found [17,18]. The autoantibodies from drug-induced pemphigus have the same antigenic specificity as in other pemphigus

Pemphigus subtype	IgG antibody	
	Anti-desmoglein 1	Anti-desmoglein 3
Pemphigus foliaceus (PF)	Yes	No
- pemphigus erythematosus	Yes	No
- fogo selvagem	Yes	No
Pemphigus vulgaris (PV)		
- mucosal	No	Yes
- mucocutaneous	Most times	Yes
- pemphigus vegetans	Sometimes	Yes
- pemphigus herpetiformis	Yes	Yes
Paraneoplastic pemphigus	Sometimes	Most times
	plectin, desmoplakin I-II, envoplakin, periplakin, BP230, 170 kDa molecule	
Drug-induced pemphigus	Most times	Most times
IgA pemphigus	IgA antibody	
- subcorneal pustular dermatosis	Desmocollin 1	
- intraepidermal neutrophilic IgA dermatosis	Unknown	

Table 1. Subtypes of pemphigus.

patients [17,19].

In IgA pemphigus, not IgG, but IgA autoantibodies are directed against the cell surface of the keratinocytes. This variant of pemphigus presents typically by pustules that have a tendency to coalesce to later form annular and circinate lesions. Several desmosomal components are recognized as target antigens, including desmocollin 1 and 2. There are two subtypes in this category: intraepidermal neutrophilic IgA dermatosis, characterized by intraepidermal pustules and subcorneal pustular dermatosis, characterized by subcorneal pustules. Desmocollin 1 is identified as autoantigen of the subcorneal pustular dermatosis type [20], while the autoantigen of the intraepidermal neutrophilic dermatosis type until now has remained unknown although sporadically a reaction to Dsg has been found [21,22,23].

Desmogleins as pemphigus antigens

The isolation of complementary DNA (cDNA) clones that encoded the pemphigus antigens has been a major progress in the investigation of pemphigus. In 1990, Koch *et al.* isolated the cDNA clone of Dsg1 from bovine muzzle epithelium. Thereafter, cDNA for human Dsg1 from human stratified squamous epithelia or cultured keratinocytes was discovered [24-26]. In 1991 Amagai *et al.* isolated cDNA for the PV antigen with autoantibodies from PV patients' sera [27]. This PV antigen was then termed desmoglein 3 (Dsg3) because this antigen was more closely related to Dsg1 than previously characterized other cadherins.

It is still a subject of debate if blistering is caused by anti-Dsg3 antibodies or if other non-Dsg antibodies also contribute to the observed pathogenicity of pemphigus patient serum IgG [28] (see also discussion on non-desmoglein targets below). Nonetheless, it is clear

that anti-Dsg3 antibodies have pathogenic properties and also exert more effects than only hampering desmosome trans-adhesion by competing for the intermolecular binding sites. While results from older studies with PV sera may indeed be challenged on grounds of sera not being proven to be pure anti-Dsg3, such is not the case for experiments with anti-Dsg3 monoclonal antibodies. Tsunoda *et al.* induced blisters in neonatal mice using two different anti-Dsg3 monoclonals [29]. The pathogenicity of such monoclonals was also shown in an *in vitro* dissociation assay [30]. Furthermore Yamamoto *et al.* recently demonstrated that depletion of Dsg3 from the membrane pools and desmosomes of DJM-1 cells, as initially demonstrated for PV sera, could also be evoked by anti-Dsg3 monoclonals [31-33].

Non-desmoglein proteins as possible pemphigus antigens

Immunoelectron studies demonstrated that PV autoantibodies bind not only to the extracellular domain of desmosomes but also along large portions of keratinocytes outside desmosomal structures [34,35]. In 2000 Nguyen *et al.* demonstrated that PV sera displayed an intercellular staining in the epidermis of Dsg3 -/- mice [36]. This suggests that keratinocyte proteins other than Dsg1 and Dsg3 could also be target antigens for PV IgG and that the immunological targets in PV are more heterogeneous than estimated. In 2003 Nguyen *et al.* demonstrated that keratinocyte cholinergic receptors can regulate desmosomal adhesion by altering the expression levels of both Dsg1 and Dsg3 and phosphorylation status of Dsg3 [37]. Several studies appeared on the possible involvement of the epithelial cholinergic system in PV. In cell studies it was shown that activation of acetylcholine receptors affected the signaling pathway that regulated the function of adhesion molecules [38,39]. Furthermore, acantholysis inducing antibodies recognized the 9-acetylcholine receptor, a 50-kDa homopentamer that regulates adhesion in the epithelial cells, and pemphaxin, a 75- kDa novel annexin that acts as an acetylcholine receptor [40]. Another study demonstrated an improvement and/or prevention of the acantholysis with cholinomimetics drugs such as carbachol and pyridostigmine bromide [41]. These data suggest that somehow the acetylcholine and its receptors might contribute to the pathogenesis of acantholysis. However definitive proof that antibodies to pemphaxin and the 9-acetylcholine receptor really exist still has to be supplied.

Sporadically IgG antibodies to desmocollin are reported. Most times these are present in addition to anti-desmoglein antibodies. An exception forms the case report of Bolling *et al.* in which a patient is described with epidermal acantholysis and anti-desmocollin 3 antibodies in the absence of anti-desmoglein antibodies [42]. This report is important as it shows that IgG mediated pemphigus can occur without anti-desmoglein antibodies. Before discussing further the pathogenesis of pemphigus, we will briefly describe the desmosomes and the structure to better understand how different autoantibodies can cause acantholysis.

Desmosomes

Desmosomes are intercellular adhesion structures that link the intermediate filament network of one cell to that of its neighbours and provide intercellular binding in many tissues

that experience stress, including heart and skin. Desmosomes were for the first time observed by Giulio Bizzozero in 1846 and described as small dense nodules which were then named "nodes of Bizzozero". The term "desmosome", suggested by Josef Schaffer in 1920, is derived from the Greek words "desmos" that means bond and "soma" that means body. Morphologically, the desmosome is divided into three zones: the extracellular core region, the outer dense plaque (ODP) and the inner dense plaque (IDP) [43-47]. These zones are occupied by two groups of proteins: transmembrane glycoproteins and intracytoplasmic plaque proteins.

The transmembrane glycoproteins comprise desmogleins and desmocollins. These proteins are from subfamilies of the cadherin superfamily of calcium dependent adhesion molecules. The extracellular domains of the desmogleins and desmocollins mediate the direct adhesion in the intercellular space of desmosomes. These transmembrane glycoproteins bind through homophilic interaction with their opposite transmembrane N-terminal domains. The cytoplasmic tails of these cadherins occupy the outer dense plaque where they bind to members of the armadillo and plakin family proteins [43,47,48]. Plakoglobin, an armadillo protein member, binds directly to the cytoplasmic tails of desmogleins and desmocollins [49,50], while desmoplakin, a plakin family member, interacts with plakoglobin and another armadillo family protein member, plakophilins [51]. In the end, desmoplakin binds the keratin filament and this forms the inner dense plaque, joining the cytoskeletal network to the desmosomal complex [43,47,48].

Cadherins

Cadherins are calcium-dependent cell-cell adhesion molecules. Their name is derived from calcium and the verb "adhere". They are divided into 2 major subgroups: classic cadherins, such as E-, P-, N-, V-cadherins and desmosomal cadherins (desmogleins and desmocollins). The classic cadherins are found at the adherens junction, another major adhering junction where the actin microfilaments anchor, and that mediates rapid but weak cell adhesion, while the desmosomal cadherins are located in the desmosome and mediate slow but strong adhesion.

The desmosomal cadherins consist of desmogleins and desmocollins. There are four isoforms of desmoglein (Dsg 1-4) and three isoforms of desmocollin (Dsc 1-3) [52]. Both groups contain four extracellular cadherin homology repeats that are approximately 110 amino acids long and a domain termed the extracellular anchor [53]. The transmembrane domain is followed by an intracellular anchor (IA) at the cytoplasmic site of the plasma membrane [52,54]. The desmocollins gene products are alternatively spliced, resulting in a Dsc "a" form and a shorter Dsc "b" form, which differ in their carboxy-terminal domain lengths [55,56]. Furthermore, the desmogleins and the Dsc "a" contain an intercellular cadherin-like sequence, which binds plakoglobin [57-59]. The desmogleins harbour an intracellular proline-rich linker (IPL) domain, a number of repeat unit domains (RUD) and a glycin-rich desmoglein terminal domain (DTD) [47]. As said before cadherins bind each other with their opposite transmembrane N-terminal domains. Although assumed that they display homophilic interaction there are

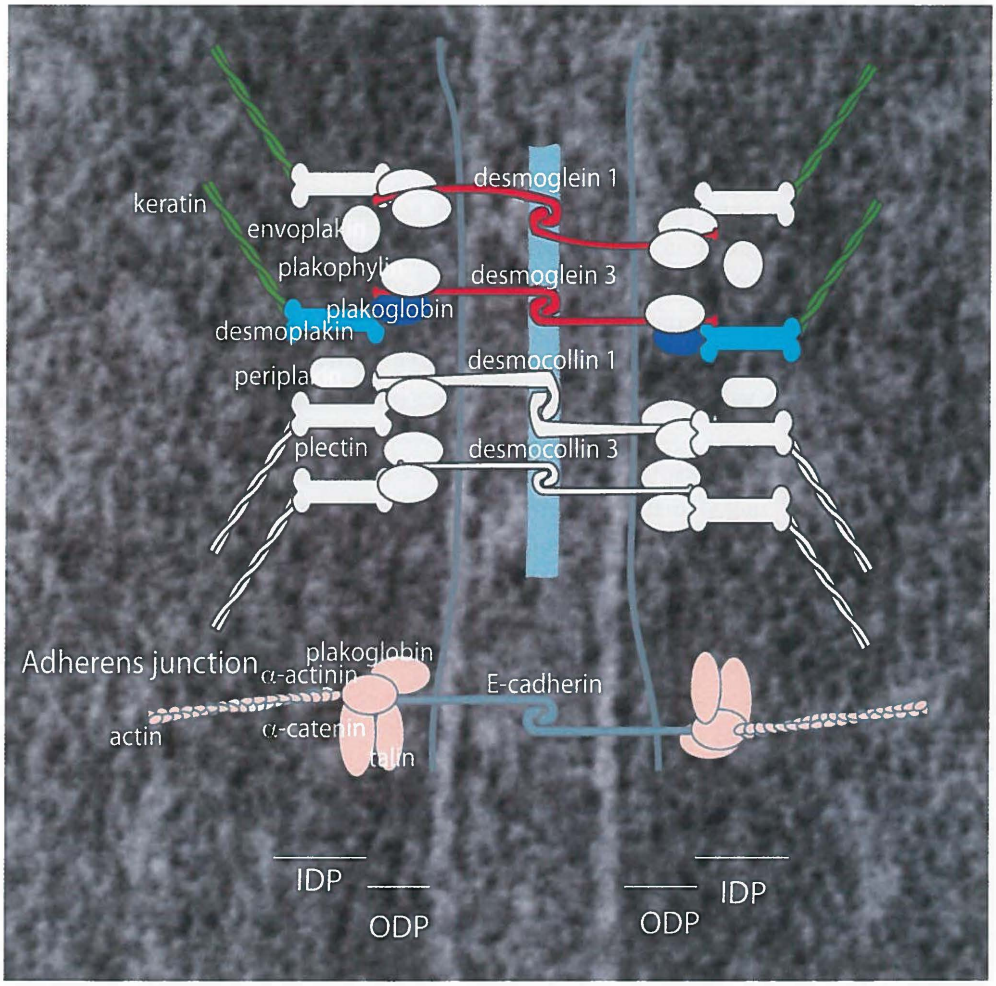


Figure 4. The schematic picture of a desmosome is overlaid on the electron microscope picture of a desmosome.

also suggestions that there may also be heterophilic interactions [60]. However in 2005 Waschke *et al.* suggested that the desmogleins are mostly mediated by the homophilic interaction [61].

In the epidermis, all seven desmosomal cadherins can be expressed. Dsg1 is distributed throughout the epidermis with the most intense expression in the upper layers, while Dsg3 is expressed most strongly in the lower layers. Under normal conditions Dsg2 is not detectable at the protein level but can be induced and is then present in the basal layer. Dsg4 is mainly expressed in the granular layer and hair follicle. Dsc 1 is expressed in the granular layer, while Dsc2 and Dsc3 are expressed most strongly in the basal layer with a decreasing expression in the higher layers [62,63].

Armadillo family proteins

These proteins play an important role to connect the desmoplakin and keratin filament network to the desmosomal complex. They are the primary link between desmoplakin and the

cytoplasmic domain of the cadherins. It is also thought that these proteins regulate the recruitment of the desmosomal components and furthermore mediate important signal transduction pathways [64]. Three subfamilies are recognized.

Classical armadillo family proteins

These are beta-catenin and plakoglobin (also known as gamma-catenin). Beta-catenin is located in the adherens junction and interacts with E-cadherin. Plakoglobin structurally contains 12-arms repeats flanked by distinct amino- and carboxy-terminal domains [47,65,66]. Plakoglobin localizes to desmosome and adherens junctions, but its affinity for the desmosomal cadherins is several times greater than for E-cadherins [67]. The central armadillo domain of plakoglobin interacts with desmoplakin that connects intermediate filaments to the desmosomal plaque.

Several studies suggest that plakoglobin plays a critical role in desmosome assembly *in vivo*. Plakoglobin null animals die due to the fragility of the myocardium [68,69]. Plakoglobin null mice also showed acantholysis, indicating that the desmosome function is weakened [70].

P-120^{ctn} related proteins

These are p120^{ctn}, ARVCF, delta-catenin (NPRAP), and p0071 (plakophilin 4). All these are also involved in adhesion and signal transduction. As these are less essential for this thesis they will not be discussed any further here.

Plakophilins

Structurally, plakophilins (PKPs) contain 9 arm repeat domains. Different from p120-catenin, these proteins are mainly found at the desmosomes instead of the adherens junctions. There are three isoforms of this group: PKP1, PKP2 and PKP3. Of PKP1 and PKP2 two forms exist, a short "a" form and a longer "b" form. PKP1 is highly expressed in the suprabasal layers of the stratified epithelia, while PKP2 is expressed in simple epithelia, the lower layers of stratified epithelia and in non epithelial tissues such as cardiac muscle and lymph nodes. PKP3 exists in simple and stratified epithelia and its distribution is uniform throughout the epidermis [71-76]. PKP3 interacts with the largest number of desmosomal proteins, such as desmoplakin, plakoglobin, desmogleins, Dsc3a and Dsc 3b, Dsc1a and Dsc2a [77]. The recent studies of Green *et al.* suggested that PKP2 plays an important role in transport of desmoplakin to the plasma membrane during desmosome assembly. They demonstrated also that PKP2 is a scaffold for PKC-α and thereby regulates desmoplakin binding with the intermediate filament [63].

Plakin family proteins

The plakin protein family consists of seven proteins which are desmoplakins I and II, envoplakin, BP230, periplakin, epiplakin, MACF and plectin (for review see Sonneberg and Liem Experimental Cell Research 2007) [78].

The member that is most relevant to this thesis is desmoplakin. Desmoplakin belongs to the plakin family of cytolinkers [78,79]. These proteins connect cytoskeletal networks to the plasma membrane and join actin, microtubules, and intermediate filaments. Structurally, desmoplakin holds globular amino- and carboxy-terminals, connected by a central α-helical coiled-coil rod domain. The amino-terminal domain supplies binding sites for plakoglobin and plakophillins, and thus targets the protein to the cadherin-armadillo protein binding at the desmosome [54]. The carboxy-terminal domain that contains three plakin repeat domains regulates desmoplakin binding to intermediate filaments [48,80,81]. There are three desmoplakin isoforms, DP I, DP Ia, and DP II, which are produced as a result of alternative RNA splicing [82]. All isoforms are expressed in numerous tissues [83]. DP II is distributed in simple epithelia [84]. Desmoplakin holds an important role since mice lacking desmoplakin die shortly after the implantation and show fewer desmosomes compared to the wild type. The observed desmosome in desmoplakin null embryos do not attach to the intermediate filaments. These data demonstrated the importance of desmoplakin in linking intermediate filaments to the plasma membrane [85,86]. The other plakins also all function in linking cytoskeletal elements together and to connect them to junctional complexes, but are less essential for the work in this thesis and will not be discussed any further here.

The formation and dynamics of desmosomes

The dynamics of desmosome formation has so far been mainly studied in cultured cells. For lack of data on actual skin therefore we have to rely on cell-derived concepts. Currently it is assumed that cadherins are constitutively synthesized and transported to the cell membrane in vesicles from the Golgi apparatus. On cell-cell contact cadherins, and possible associated plaque proteins suchas plakoglobin, cluster and non-membranous particles containing desmoplakin and plakophilin, that move along the intermediate filaments towards the cell-junctions, are added. Together these can nucleate a new desmosome that grows when more plaque molecules are incorporated, a process that seems to depend on plakoglobin and plakophilin (for reviews see [60,63]). Time-lapse studies on epithelial A-431 cells showed a continuous appearance of small cadherin and plakoglobin containing 'puncta' in the membrane that could fuse into a small desmosome. Moreover such puncta were also incorporated into already established desmosomes which suggests that desmosomal cadherins are continuously replaced by newly synthesized molecules [87]. This implies that also the cytoskeleton has to be restructured continuously, what indeed has been observed [88]. The overall picture is that of a desmosome that continuously rejuvenates itself. Having seen that the involved cadherins are also the autoantigens in pemphigus, this process of rejuvenation may become disturbed in pemphigus.

Pathogenesis of pemphigus

The basic molecular mechanism of the blister formation in pemphigus is acantholysis, loss of cell-cell adhesion between keratinocytes, which is caused by autoantibodies against the

intercellular adhesion molecules of the keratinocytes. It is already known that most of the autoantibodies in patients with active PV are from the IgG4 subclass [89,90], while in patients in remission they are more often of the IgG1 subtype. The healthy relatives and healthy carriers of PV-prevalent HLA class II alleles carry low levels of IgG1 autoantibodies [90-92].

Target antigens known are desmosomal components Dsg1 in PF and Dsg3, sometimes in combination with Dsg1, in PV [27,93-95]. The PV autoantibodies target specifically the extracellular (EC) domain of Dsg3 [89,96]. The IgG from PV sera affinity-purified on the EC1-2 of Dsg3 causes suprabasal acantholysis, the typical histological finding of PV. However IgG affinity-purified on a recombinant protein representing the EC3-5 of Dsg3 could not induce acantholysis when injected into neonatal mice [96]. Furthermore, IgG1 and IgG4 from patients with active PV identify epitopes in the EC1 and EC2. *In vitro* data indicated that IgG4 directed against the EC2 and in a lesser extent the EC1 caused acantholysis [89]. These data suggest that IgG4 directed to the EC2 of Dsg3 has the main acantholytic property, while IgG4 directed to the EC1 of Dsg3 may have an enhancing effect on this process.

The IgG immunoglobulin fraction of the patient serum is sufficient to produce acantholysis in various experimental models, such as cultured keratinocytes and in skin explants *in vitro* [97,98] or passive transfer experiments in neonatal mice [99]. Furthermore, IgG affinity-purified from PV sera on the extracellular domain of Dsg3 can cause suprabasal blistering when injected into neonatal mice [96]. When the anti-Dsg3 IgG was immuno-adsorbed by the extracellular domain of Dsg3 from PV sera, then these sera lost their blister inducing pathogenicity in neonatal mice [100]. Similarly immunoadsorption of PF sera with extracellular domains of Dsg1 abolished the pathogenic ability [101]. In 1997 Koch *et al.* engineered mice genetically with a target disruption of the Dsg3 gene [102]. At birth these mice were still normal but later on they developed a phenotype of oral erosions leading to weight loss due to impaired food intake. This suggests that the anti-Dsg3 autoantibody mainly induces mucosal lesions. Interestingly, these mice developed cutaneous blisters when the skin was traumatized and they developed telogen hair loss.

Various *in vivo* and *in vitro* studies on the effects of pemphigus IgG undoubtedly demonstrated the pathogenicity of pemphigus IgG [103]. However, the exact mechanism by which IgG induces loss of adhesion still remains unclear. There are several concepts that still exist until today. The first such concept is known as the steric hindrance or "direct hit" theory. This theory proposes that the binding of IgG to desmoglein may simply block and disrupt homophilic as well as heterotypic trans-interaction and thus lead to the splitting of desmosomes and loss of cell-cell adhesion [104]. However, experimental evidence to support this theory has not been produced. The strongest argument against this concept comes from a study on plakoglobin-null cells by Caldelari *et al.* [105]. They demonstrated that PV IgG is unable to induce acantholysis in these plakoglobin-null cells while at the same time it did induce acantholysis in wild-type cells [105]. They suggested that a cytoplasmic protein is needed for acantholysis and that acantholysis cannot simply occur from simple lengthwise splitting of desmosomes.

The second hypothesis assumes that the IgG binding to desmoglein activates intracellular signalling pathways and ends with cytoskeleton collapse or desmosome disassembly. Plakoglobin-deficient keratinocytes in the study of Caldelari *et al.* did not respond to pemphigus IgG, showing that the desmosome disruption is a downstream event after IgG binding to desmoglein [105]. Additionally, human keratinocytes treated with PV IgG at 4°C do not demonstrate loss of cell-cell adhesion until those cells are shifted to 37°C. This observation suggested that for antibodies to cause loss of keratinocytes adhesion a response (and energy) is needed [106].

Several studies revealed that PV IgG binding may induce phosporylation of heat shock protein (HSP) 27 through p38 mitogen-activating protein kinase (p38MAPK) and that inhibition of p38MAPK prevents keratin retraction, actin reorganization and formation of blisters in a pemphigus mouse model. The concept that disturbance of cell-signalling underlies acantholysis has been suggested both in PV and in PF, featuring roles not only for p38MAPK but also for RhoA [107-111]. Signalling via the *c-Myc* pathway has also been hypothesized to play a role in PV pathogenesis [112]. The internalization of plakoglobin (PG), although not leading to decreased total steady state PG levels, does lead to lower nuclear PG levels [112]. As PG is a transcriptional suppressor of the *c-Myc* gene, consequently an increase of nuclear c-Myc would be expected, what indeed is observed in PV skin [113]. This c-Myc is believed to be involved in PV pathogenesis and c-Myc inhibitors were shown to suppress PV pathogenesis in a mouse model system [112]. Put forward by Muller *et al.* in 2008, these ideas of a signal transduction pathway through membrane bound receptors and adhesion molecules is described as the "outside-in signalling" theory [114]. These studies suggest that the activation of the signalling pathways on autoantibody binding causes loss of adhesion, and the manipulation of the intracellular signalling pathway by selective inhibitors may prevent blistering caused by pemphigus IgG.

The third concept features the idea that IgG binding to the desmoglein causes Dsg3 to be internalized through an endo-lysosomal pathway. This event results in the loss of surface Dsg3 that parallels the disruption of keratinocyte cell adhesion. PV IgG has been demonstrated to cause internalization of the newly synthesized pools of Dsg3 [115,116]. Time-course experiments demonstrated two phases of Dsg3 depletion: a rapid phase in which the non-desmosomal Triton X-100 soluble pool is lost and a later phase in which desmosomal Dsg3 also disappears and Dsg3-depleted desmosomes appear [31]. Depletion of Dsg1 after PF IgG binding on cultured cells was observed. Interestingly, in contrast to PV only soluble Dsg1, not the Triton-insoluble Dsg1, was internalized [117]. The internalization of Dsg3 takes place in complexes of Dsg3 and IgG that were recently shown also to contain PG [106]. In cells the Dsg3 endocytosis was shown to proceed through a clathrin-independent pathway and that Dsg3 was routed into a lysosomal compartment for degradation [106,118]. As a consequence, blocking the Dsg3 endocytosis could prevent keratinocyte loss of adhesion in response to PV IgG [118]. The overall result of a decrease of the targeted cadherin is that the resulting desmosomes become depleted of it and thereby lose their adhesive strength.

A fourth concept is a variant of this. Here it is supposed that cadherin molecules that have IgG bound to it cannot be properly incorporated anymore in desmosomes what will lead to severe desmosome assembly problems. The final result is the same as in concept three but removal of the Dsg3 by endocytosis is not considered here the main cause of depletion but instead the inability of Dsg3 bound IgG to incorporate into the desmosome [119].

Autoreactive T lymphocytes in pemphigus

Although pemphigus is an antibody mediated disease, T-cells are important for the pathogenesis. Current studies suggest that autoreactive T-cells play a crucial role in the initiation of both antibody- and cell-mediated autoimmune diseases. Autoreactive T-cells provide critical help for B-cells to continuously produce pathogenic autoantibodies in PV [120]. Both Th-1 and Th-2-like Dsg3-specific T-cells were identified in PV patients [121]. While the Th2 cytokines IL-4 and IL-13 have been shown to be involved in regulation of IgG4 and IgE secretion by activated B-cells, the Th1 cytokine IFNγ induces IgG1 secretion. Both autoreactive Th1- and Th2-cells may be involved in the regulation of the production of pathogenic autoantibodies by B cells in PV as PV patient sera contain Th1-regulated IgG1 and Th2-regulated IgG4, IgA and IgE autoantibodies to desmoglein [89,90,122].

Hypotheses about why blisters occur at defined levels

One of the most intriguing questions in pemphigus is why PV patients have blisters on mucosal tissue, sometimes accompanied by skin involvement, while in PF blistering is limited to the skin. Below we present the most prominent hypotheses that attempt to explain this.

Desmoglein compensation hypothesis

In 1999 Mahoney *et al.* explained the different blister levels by what they called the desmoglein compensation hypothesis. A suggestion with a similar concept had already been made six years before, by Burge *et al.* , however that paper remained relatively unnoticed [14,123]. Mahoney *et al.* utilized a Dsg3 -/- mouse model to demonstrate the role of Dsg3 in limiting the blister formation in PF. Upon the transfer of PF IgG, Dsg3 +/+ mice developed small cutaneous blisters, while the Dsg3 -/- developed extensive blisters on the skin and mucous membranes. These observations suggest that Dsg3 inhibits blister formation in both skin and mucous membranes. This led them to the hypothesis that destabilization of desmosomes by either anti-Dsg1 or anti-Dsg3 IgG does not happen if Dsg1 and Dsg3 are simultaneously expressed, and that blistering will occur as a compensating Dsg is missing. Dsg3 is expressed throughout the oral mucosa, while in the epidermis it is expressed only in the basal and immediate suprabasal layers. In contrast, Dsg1 is expressed throughout the epidermis, with a declining expression from the higher to the lower layers, while it is hardly expressed in the mucosa. In PF anti-Dsg1 IgG causes blisters in the superficial layers of the epidermis where only Dsg1 is expressed, but not in the deep epidermis or mucosa where the Dsg3 expression compensates for the functional loss of Dsg1. In mucosal PV, anti-Dsg3 IgG will cause acan-

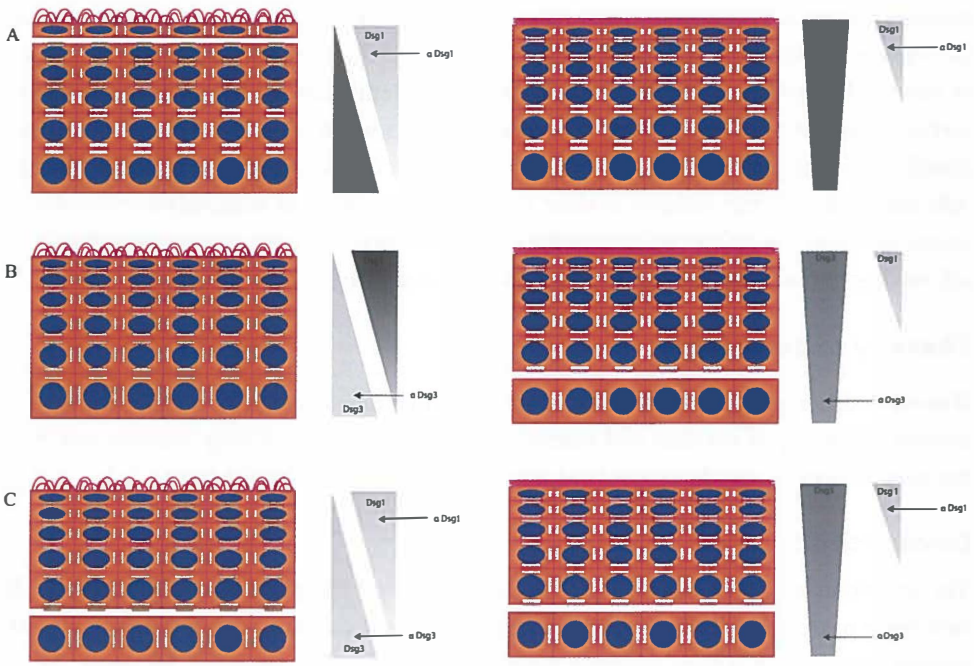


Figure 5. Schematic picture of the desmoglein compensation hypothesis. (**A**) Pemphigus foliaceus sera which contain only anti-Dsg1 IgG causes superficial blisters in the skin because Dsg3 compensates for the impaired function of Dsg1 in the lower layers of the epidermis. However, those antibodies do not cause blisters in the mucous membranes as cell–cell adhesion is mainly mediated by Dsg3. (**B**) Sera which contain only anti-Dsg3 IgG cause no or limited blisters in the skin because Dsg1 compensates for the loss function of Dsg3. However, these sera induce separation in the mucous membranes, where the low expression of Dsg1 cannot compensate for the loss of Dsg3. (**C**) When sera contain both anti-Dsg1 and anti-Dsg3 IgG, the function of both desmogleins is impaired and blisters occur in both the skin and mucous membranes.

tholysis in the deepest layers of the mucous membranes where Dsg1 expression is minimal. In mucocutaneous PV, both anti-Dsg1 and anti-Dsg3 antibodies will attack the desmosomes and result in blistering of both the skin and mucous membranes [124] (Figure 5). That in PV the blister forms suprabasal rather than subcorneal can be due to cell-cell adhesion between basal and suprabasal cells being weaker than in other parts of the epidermis as there are fewer desmosomes [62]. Alternatively, and probably a much better explanation is that the lower layers are the first layers that contact antibodies that penetrate from the dermis, and therefore are the first to undergo acantholysis.

Basal cell shrinkage theory

An alternative idea to explain why suprabasal blistering is observed in PV is the basal cell shrinkage hypothesis put forward by Bystryn and Grando [125]. The basic idea here is that basal cells express different protein repertoires than more differentiated cells inherent to their

specific role in the epithelium. Much significance is ascribed to the composition of their cytoskeletons that are built from different keratins, whereby it is assumed that basal cells have the weaker cytoskeleton, and to the types of cell surface receptors that are uniquely expressed by basal cells. Bystryn and Grando believe that PV patients have antibodies to the unique cell surface receptors of basal cells and that these receptors when bound by patient IgG activate specific signaling pathways that initiate the breakdown of the cytoskeleton. Then as the basal cells shrink due to the collapse of their cytoskeleton—visible as acantholysis—the desmosomes would not be able to hold on to the overlying suprabasal layer anymore and finally tear off, leaving the basal layer separated from the suprabasal layer.

Therapy of pemphigus

If untreated, pemphigus has severe morbidity and high mortality. Major complications of the massive blistering of the skin and mucous membranes are loss of body proteins and fluids, bacterial infections and decreased food uptake related to painful oral lesions.

Glucocorticoids

The invention of glucocorticoids dramatically improved the prognosis of pemphigus. The treatment strategy is to induce complete remission by administration of for example systemic prednisolone, which is then gradually tapered over weeks to months [3,126]. However the side effects of chronic glucocorticoid treatment such as hypertension, diabetes, osteoporosis, and increased susceptibility to infections do contribute to the mortality. Glucocorticoids are most times given in combination with adjuvants to allow a gradual tapering of the glucocorticoids.

Adjuvants

Immunosuppressive adjuvants are known as "steroid-sparing" drugs and guarantee long-lasting immunosuppression without the need for chronic use of high dose of systemic glucocorticoids. The most commonly used agents are azathioprine, cyclophosphamide, mycophenolate mofetil, and methotrexate [7].

Removal of pathogenic antibodies

Other strategies target on interfering with the pathogenic autoantibodies in pemphigus. Plasmapharesis has been used for years to remove pathogenic autoantibodies from pemphigus patient sera. Immunoadsorption has been established as an adjuvant treatment in several autoimmune diseases. There are a number of immunoadsorbent columns available for clinical purposes: protein A, tryptophan, phenylalanine and dextran sulfate are used as ligands [127]. The adsorbents work by removing the targets via hydrophobic binding. The advantages of this method over the unselected plasmapharesis are the higher selectivity in the pathogen removal, reduced loss of essential plasma components and no requirements for protein replacement with all its risks.

Rituximab

First approved as a therapy for non-Hodgkin B-cell lymphoma [128], rituximab has been used to treat patients with autoimmune bullous dermatoses, including PV and bullous pemphigoid, and other autoimmune diseases, such as systemic lupus erythematosus, idiopathic thrombocytopenic purpura, multiple sclerosis, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, and dermatomyositis [129,130]. Rituximab is a human/murine chimeric monoclonal antibody anti-CD20. It binds to CD20 on the cell surface of mature B-cells, targets and further depletes CD20+ B-cells, but not B-stem cells, pro B-cells, or existing plasma cells [131,132].

The B-cell depletion mediated by rituximab is likely to be caused by several possible mechanisms, which act individually or collectively depending on the disease pathology. Rituximab may possibly kill the B-cell by natural killer (NK) cells through antibody-dependent cellular toxicity (ADCC). Direct cross linking of CD20 on B-cell can also induce apoptosis [133], through p38 MAP kinase activation [134]. The binding of rituximab to the surface CD20 on B-cells can also induce complement dependent cytotoxicity on target B-cells and apoptosis through membrane attack complex [133-135]. Opsonization of B-cells by rituximab may also induce the B-cells clearance in the circulation through phagocytosis by reticuloendothelial system such as macrophages [136].

There is increasing evidence for the successful treatment of severe and refractory PV due to rituximab's B-cell depleting ability [137-139]. Dramatic clinical responses are also reported in trials using rituximab in combination with systemic glucocorticoids or with the high dose intravenous immunoglobulin [140-142]. Eming *et al.* studied the Dsg3-specific Th1 and Th2 cells of the PV patients treated with rituximab and demonstrated that there was a significant decrease of autoreactive Dsg3-specific CD4+ Th cells and much later a significant decrease of autoantibody against Dsg3 [143]. They suggested that the response of pemphigus vulgaris to rituximab involves dual effects, which are the depletion of the autoreactive B-cells and the downregulation of Dsg3-specific CD4+ Th-cells.

Recently rituximab was approved by the Food and Drug Administration (FDA) for rheumatoid arthritis [144], after its approval for the lymphoma treatment [145]. However, the use of rituximab in the treatment of autoimmune blister diseases is still off-label. Rituximab has been considered mainly in adjuvant setting in pemphigus [137]. The optimal combination with additional immunosuppressive drugs or high dose intravenous immunoglobulin [141] or immunoadsorption [146] still needs to be defined. More clinical trials need to be done in order for rituximab to be the first choice for the treatment of autoimmune blister diseases.

Aims of the study

The overall aim of this study is to contribute to unravelling the pathogenesis of pemphigus. In contrast to the mainstream of pemphigus research, which is done in cultured cell or in mouse models, we chose to study pemphigus from where the actual pathological events take place, i.e. patient skin. We made this choice as we realized that models may well unravel aspects of the disease but will never show the actual pathogenesis as they are different from human skin.

Pemphigus pathogenesis

Pemphigus patients generate autoimmune immunoglobulin that binds to specific molecules of the epidermis. Skin of patients therefore demonstrates immune deposition at the intercellular space of the epidermis. When serum of a patient is brought onto a section of epithelium such as monkey oesophagus or skin, serum pemphigus antibodies will bind to the epithelial cells in a similarly intercellular pattern [147]. However, the *in vivo* deposition pattern of pemphigus antibodies is different from the pattern of the serum antibodies bound to normal skin [92,148]. The ICS pattern is a smooth staining around keratinocytes, also called "honey-comb" or "chicken wire" pattern, and is in line with the normal distribution of the Dsg's. In patient skin however the IgG deposits have a granular appearance [123]. Many *in vitro* studies have demonstrated the pathogenicity of pemphigus IgG in inducing acantholysis. However, very few studies have been performed on patient skin biopsies to observe if the same phenomenon exists *in vivo*. In **chapter 2** of this thesis we investigated multiple pemphigus patient skin biopsies in order to understand why the IgG deposits *in vivo* in such an aberrant granular fashion in both PV and PF, and if this is related with acantholysis.

In the following **chapter 3** we have investigated the same phenomenon mechanism but now in oral mucosal tissue. The desmosomes of mucosal keratinocytes have another cadherin composition than the keratinocytes of the epidermis. Acantholysis in mucosal keratinocytes occurs in patients with IgG against Dsg3 but not in patients with IgG against Dgs1. Therefore mucosal tissue is the only tissue in which acantholysis that is anti-Dsg3 driven can be studied.

In **chapter 4** we address a subform of PF that is known as pemphigus erythematosus (PE). These patients have in sun-exposed skin a granular deposition of IgG along the basement membrane, similar to patients with chronic discoid lupus erythematosus (CDLE) and subacute cutaneous lupus erythematosus (SCLE). For this reason PE long has been considered a combination of PF and LE. We have analyzed the composition of the lupus band and based on that we conclude that PE is in fact a photosensitive subform of PF.

In **chapter 5** we have analyzed our biopsies to investigate if endocytosis of desmosomes or desmosomal components takes place in pemphigus patient skin. Endocytosis, as discussed earlier, is a prominent part of the main hypotheses on acantholysis. In experimental cell systems endocytosis of IgG and cadherins has convincingly been demonstrated. However nobody so far has verified if such endocytosis also takes place in patient skin.

In **chapter 6** we study the fate of Dsg1 in skin of patients with Staphylococcus Scalded Skin Syndrome (SSSS). SSSS is a disease which shows generalized and superficial exfoliative lesions similar to PF. SSSS occurs mostly in neonates and children. However also adults with renal failure and immune compromised individuals are at risk [104]. *In vitro* studies predict that blistering is due to Staphylococcus aureus exfoliative toxins that would cleave off the extracellular domain of Dsg1. However such hypothesis has never been tested by actual patient skin. Therefore we have investigated the fate of Dsg1 in skin samples of eight SSSS patients.

References

1 Bystryn JC, Rudolph JL. Pemphigus. *Lancet* 2005; **366**: 61-73.

2 Chams-Davatchi C, Valikhani M, Daneshpazhooh M *et al.* Pemphigus: analysis of 1209 cases. *International journal of dermatology* 2005; **44**: 470-6.

3 Nousari HC, Anhalt GJ. Pemphigus and bullous pemphigoid. *Lancet* 1999; **354**: 667-72.

4 Hertl M. Humoral and cellular autoimmunity in autoimmune bullous skin disorders. *International archives of allergy and immunology* 2000; **122**: 91-100.

5 Ishii K, Amagai M, Komai A *et al.* Desmoglein 1 and desmoglein 3 are the target autoantigens in herpetiform pemphigus. *Archives of dermatology* 1999; **135**: 943-7.

6 Jablonska S, Chorzelski TP, Beutner EH *et al.* Herpetiform pemphigus, a variable pattern of pemphigus. *International journal of dermatology* 1975; **14**: 353-9.

7 Hertl M. Pemphigus. In: *Autoimmune Diseases of the Skin* (Hertl M, ed), 2nd edn. Wien, New York: Springer. 2005.

8 Baykal C, Azizlerli G, Thoma-Uszynski S *et al.* Pemphigus vulgaris localized to the nose and cheeks. *Journal of the American Academy of Dermatology* 2002; **47**: 875-80.

9 Abida O, Masmoudi A, Rebai A *et al.* The familial feature of Tunisian endemic pemphigus foliaceus. *The British journal of dermatology* 2009; **161**: 951-3.

10 Anhalt GJ, Kim SC, Stanley JR *et al.* Paraneoplastic pemphigus. An autoimmune mucocutaneous disease associated with neoplasia. *The New England journal of medicine* 1990; **323**: 1729-35.

11 Oursler JR, Labib RS, Ariss-Abdo L *et al.* Human autoantibodies against desmoplakins in paraneoplastic pemphigus. *The Journal of clinical investigation* 1992; **89**: 1775-82.

12 Kim SC, Kwon YD, Lee IJ *et al.* cDNA cloning of the 210-kDa paraneoplastic pemphigus antigen reveals that envoplakin is a component of the antigen complex. *The Journal of investigative dermatology* 1997; **109**: 365-9.

13 Kazerounian S, Mahoney MG, Uitto J *et al.* Envoplakin and periplakin, the paraneoplastic pemphigus antigens, are also recognized by pemphigus foliaceus autoantibodies. *The Journal of investigative dermatology* 2000; **115**: 505-7.

14 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *The Journal of clinical investigation* 1999; **103**: 461-8.

15 Joly P, Gilbert D, Thomine E *et al.* Immunofluorescence and immunoelectron microscopy analyses of a human monoclonal anti-epithelial cell surface antibody that recognizes a 185-kD polypeptide: a component of the paraneoplastic pemphigus antigen complex? *The Journal of investigative dermatology* 1993; **101**: 339-45.

16 Proby C, Fujii Y, Owaribe K *et al.* Human autoantibodies against HD1/plectin in paraneoplastic pemphigus. *The Journal of investigative dermatology* 1999; **112**: 153-6.

17 Korman NJ, Eyre RW, Zone J *et al.* Drug-induced pemphigus: autoantibodies directed against the pemphigus antigen complexes are present in penicillamine and captopril-induced pemphigus. *The Journal of investigative dermatology* 1991; **96**: 273-6.

18 Brenner S, Bialy-Golan A, Anhalt GJ. Recognition of pemphigus antigens in drug-induced pemphigus vulgaris and pemphigus foliaceus. *Journal of the American Academy of Dermatology* 1997; **36**: 919-23.

19 Penas PF, Buezo GF, Carvajal I *et al.* D-penicillamine-induced pemphigus foliaceus with autoantibodies to desmoglein-1 in a patient with mixed connective tissue disease. *Journal of the American Academy of Dermatology* 1997; **37**: 121-3.

20 Hashimoto T, Kiyokawa C, Mori O *et al.* Human desmocollin 1 (Dsc1) is an autoantigen for the subcorneal pustular dermatosis type of IgA pemphigus. *The Journal of investigative dermatology* 1997; **109**: 127-31.

21 Karpati S, Amagai M, Liu WL *et al.* Identification of desmoglein 1 as autoantigen in a patient with intraepidermal neutrophilic IgA dermatosis type of IgA pemphigus. *Experimental dermatology* 2000; **9**: 224-8.

22 Hashimoto T, Komai A, Futei Y *et al.* Detection of IgA autoantibodies to desmogleins by an enzyme-linked immunosorbent assay: the presence of new minor subtypes of IgA pemphigus. *Archives of dermatology* 2001; **137**: 735-8.

23 Tajima M, Mitsuhashi Y, Irisawa R *et al.* IgA pemphigus reacting exclusively to desmoglein 3. *Eur J Dermatol* 2010; **20**: 626-9.

24 Goodwin L, Hill JE, Raynor K *et al.* Desmoglein shows extensive homology to the cadherin family of cell adhesion molecules. *Biochemical and biophysical research communications* 1990; **173**: 1224-30.

25 Nilles LA, Parry DA, Powers EE *et al.* Structural analysis and expression of human desmoglein: a cadherin-like component of the desmosome. *Journal of cell science* 1991; **99 (Pt 4)**: 809-21.

26 Wheeler GN, Parker AE, Thomas CL *et al.* Desmosomal glycoprotein DGI, a component of intercellular desmosome junctions, is related to the cadherin family of cell adhesion molecules. *Proceedings of the National Academy of Sciences of the United States of America* 1991; **88**: 4796-800.

27 Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67**: 869-77.

28 Amagai M, Ahmed AR, Kitajima Y *et al.* Are desmoglein autoantibodies essential for the immunopathogenesis of pemphigus vulgaris, or just "witnesses of disease"? *Experimental dermatology* 2006; **15**: 815-31.

29 Tsunoda K, Ota T, Aoki M *et al.* Induction of pemphigus phenotype by a mouse monoclonal antibody against the amino-terminal adhesive interface of desmoglein 3. *J Immunol* 2003; **170**: 2170-8.

30 Ishii K, Harada R, Matsuo I *et al.* In vitro keratinocyte dissociation assay for evaluation of the pathogenicity of anti-desmoglein 3 IgG autoantibodies in pemphigus vulgaris. *The Journal of investigative dermatology* 2005; **124**: 939-46.

31 Aoyama Y, Kitajima Y. Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3-depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells. *The Journal of investigative dermatology* 1999; **112**: 67-71.

32 Yamamoto Y, Aoyama Y, Shu E *et al.* Anti-desmoglein 3 (Dsg3) monoclonal antibodies deplete desmosomes of Dsg3 and differ in their Dsg3-depleting activities related to pathogenicity. *The Journal of biological chemistry* 2007.

33 Yamamoto Y, Aoyama Y, Shu E *et al.* Anti-desmoglein 3 (Dsg3) monoclonal antibodies deplete desmosomes of Dsg3 and differ in their Dsg3-depleting activities related to pathogenicity. *The Journal of biological chemistry* 2007; **282**: 17866-76.

34 Bedane C, Prost C, Thomine E *et al.* Binding of autoantibodies is not restricted to desmosomes in pemphigus vulgaris: comparison of 14 cases of pemphigus vulgaris and 10 cases of pemphigus foliaceus studied by western immunoblot and immunoelectron microscopy. *Arch Dermatol Res* 1996; **288**: 343-52.

35 Joly P, Gilbert D, Thomine E *et al.* Identification of a new antibody population directed against a desmosomal plaque antigen in pemphigus vulgaris and pemphigus foliaceus. *The Journal of investigative dermatology* 1997; **108**: 469-75.

36 Nguyen VT, Ndoye A, Shultz LD *et al.* Antibodies against keratinocyte antigens other than desmogleins 1 and 3 can induce pemphigus vulgaris-like lesions. *The Journal of clinical investigation* 2000; **106**: 1467-79.

37 Nguyen VT, Arredondo J, Chernyavsky AI *et al.* Keratinocyte acetylcholine receptors regulate cell adhesion. *Life sciences* 2003; **72**: 2081-5.

38 Williams DM. Non-infectious diseases of the oral soft tissue: a new approach. *Advances in dental research* 1993; **7**: 213-9.

39 Conroy WG, Ogden LF, Berg DK. Cluster formation of alpha7-containing nicotinic receptors at interneuronal interfaces in cell culture. *Neuropharmacology* 2000; **39**: 2699-705.

40 Nguyen VT, Ndoye A, Grando SA. Novel human alpha9 acetylcholine receptor regulating keratinocyte adhesion is targeted by Pemphigus vulgaris autoimmunity. *The American journal of pathology* 2000; **157**: 1377-91.

41 Grando SA, Kist DA, Qi M *et al.* Human keratinocytes synthesize, secrete, and degrade acetylcholine. *The Journal of investigative dermatology* 1993; **101**: 32-6.

42 Bolling MC, Mekkes JR, Goldschmidt WF *et al.* Acquired palmoplantar keratoderma and immunobullous disease associated with antibodies to desmocollin 3. *The British journal of dermatology* 2007; **157**: 168-73.

43 Kowalczyk AP, Stappenbeck TS, Parry DA *et al.* Structure and function of desmosomal transmembrane core and plaque molecules. *Biophysical chemistry* 1994; **50**: 97-112.

44 Schmidt A, Heid HW, Schafer S *et al.* Desmosomes and cytoskeletal architecture in epithelial differentiation: cell type-specific plaque components and intermediate filament anchorage. *European journal of cell biology* 1994; **65**: 229-45.

45 Green KJ, Jones JC. Desmosomes and hemidesmosomes: structure and function of molecular components. *Faseb J* 1996; **10**: 871-81.

46 North AJ, Bardsley WG, Hyam J *et al.* Molecular map of the desmosomal plaque. *Journal of cell science* 1999; **112 (Pt 23)**: 4325-36.

47 Garrod D, Chidgey M. Desmosome structure, composition and function. *Biochimica et biophysica acta* 2008; **1778**: 572-87.

48 Getsios S, Amargo EV, Dusek RL *et al.* Coordinated expression of desmoglein 1 and desmocollin 1 regulates intercellular adhesion. *Differentiation; research in biological diversity* 2004; **72**: 419-33.

49 Wahl JK, Sacco PA, McGranahan-Sadler TM *et al.* Plakoglobin domains that define its association with the desmosomal cadherins and the classical cadherins: identification of unique and shared domains. *Journal of cell science* 1996; **109 (Pt 5)**: 1143-54.

50 Witcher LL, Collins R, Puttagunta S *et al.* Desmosomal cadherin binding domains of plakoglobin. *The Journal of biological chemistry* 1996; **271**: 10904-9.

51 Cowin P, Burke B. Cytoskeleton-membrane interactions. *Current opinion in cell biology* 1996; **8**: 56-65.

52 Green KJ, Simpson CL. Desmosomes: new perspectives on a classic. *The Journal of investigative dermatology* 2007; **127**: 2499-515.

53 Pokutta S, Weis WI. Structure and mechanism of cadherins and catenins in cell-cell contacts. *Annual review of cell and developmental biology* 2007; **23**: 237-61.

54 Kowalczyk AP, Bornslaeger EA, Norvell SM *et al.* Desmosomes: intercellular adhesive junctions specialized for attachment of intermediate filaments. *International review of cytology* 1999; **185**: 237-302.

55 Collins JE, Legan PK, Kenny TP *et al.* Cloning and sequence analysis of desmosomal glycoproteins 2 and 3 (desmocollins): cadherin-like desmosomal adhesion molecules with heterogeneous cytoplasmic domains. *The Journal of cell biology* 1991; **113**: 381-91.

56 Parker AE, Wheeler GN, Arnemann J *et al.* Desmosomal glycoproteins II and III. Cadherin-like junctional molecules generated by alternative splicing. *The Journal of biological chemistry* 1991; **266**: 10438-45.

57 Mathur M, Goodwin L, Cowin P. Interactions of the cytoplasmic domain of the desmosomal cadherin Dsg1 with plakoglobin. *The Journal of biological chemistry* 1994; **269**: 14075-80.

58 Roh JY, Stanley JR. Plakoglobin binding by human Dsg3 (pemphigus vulgaris antigen) in keratinocytes requires the cadherin-like intracytoplasmic segment. *The Journal of investigative dermatology* 1995; **104**: 720-4.

59 Troyanovsky SM, Troyanovsky RB, Eshkind LG *et al.* Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation. *Proceedings of the National Academy of Sciences of the United States of America* 1994; **91**: 10790-4.

60 Waschke J. The desmosome and pemphigus. *Histochemistry and cell biology* 2008; **130**: 21-54.

61 Waschke J, Bruggeman P, Baumgartner W *et al.* Pemphigus foliaceus IgG causes dissociation of desmoglein 1-containing junctions without blocking desmoglein 1 transinteraction. *The Journal of clinical investigation* 2005; **115**: 3157-65.

62 Delva E, Tucker DK, Kowalczyk AP. The desmosome. *Cold Spring Harbor perspectives in biology* 2009; **1**: a002543.

63 Desai BV., Harmon RM, Green KJ. Desmosomes at a glance. *Journal of cell science* 2009; **122**: 4401-7.

64 Delva E, Tucker DK, Kowalczyk AP. The desmosome. *Cold Spring Harbor perspectives in biology* 2009; **1**: a002543.

65 Peifer M, McCrea PD, Green KJ *et al.* The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *The Journal of cell biology* 1992; **118**: 681-91.

66 Huber O, Krohn M, Kemler R. A specific domain in alpha-catenin mediates binding to beta-catenin or plakoglobin. *Journal of cell science* 1997; **110 (Pt 15)**: 1759-65.

67 Chitaev NA, Leube RE, Troyanovsky RB *et al.* The binding of plakoglobin to desmosomal cadherins: patterns of binding sites and topogenic potential. *The Journal of cell biology* 1996; **133**: 359-69.

68 Bierkamp C, McLaughlin KJ, Schwarz H *et al.* Embryonic heart and skin defects in mice lacking plakoglobin. *Developmental biology* 1996; **180**: 780-5.

69 Ruiz P, Brinkmann V, Ledermann B *et al.* Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart. *The Journal of cell biology* 1996; **135**: 215-25.

70 Acehan D, Petzold C, Gumper I *et al.* Plakoglobin is required for effective intermediate filament anchorage to desmosomes. *The Journal of investigative dermatology* 2008; **128**: 2665-75.

71 Bonne S, van Hengel J, Nollet F *et al.* Plakophilin-3, a novel armadillo-like protein present in nuclei and desmosomes of epithelial cells. *Journal of cell science* 1999; **112 (Pt 14)**: 2265-76.

72 Schmidt A, Langbein L, Pratzel S *et al.* Plakophilin 3--a novel cell-type-specific desmosomal plaque protein. *Differentiation; research in biological diversity* 1999; **64**: 291-306.

73 Heid HW, Schmidt A, Zimbelmann R *et al.* Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). *Differentiation; research in biological diversity* 1994; **58**: 113-31.

74 Mertens C, Kuhn C, Franke WW. Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. *The Journal of cell biology* 1996; **135**: 1009-25.

75 Schmidt A, Langbein L, Rode M *et al.* Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components. *Cell and tissue research* 1997; **290**: 481-99.

76 Franke WW, Schumacher H, Borrmann CM *et al.* The area composita of adhering junctions connecting heart muscle cells of vertebrates - III: assembly and disintegration of intercalated disks in rat cardiomyocytes growing in culture. *European journal of cell biology* 2007; **86**: 127-42.

77 Hatzfeld M. Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? *Biochimica et biophysica acta* 2007; **1773**: 69-77.

78 Sonnenberg A, Liem RK. Plakins in development and disease. *Experimental cell research* 2007; **313**: 2189-203.

79 Green KJ, Virata ML, Elgart GW *et al.* Comparative structural analysis of desmoplakin, bullous pemphigoid antigen and plectin: members of a new gene family involved in organization of intermediate filaments. *International journal of biological macromolecules* 1992; **14**: 145-53.

80 Choi HJ, Park-Snyder S, Pascoe LT *et al.* Structures of two intermediate filament-binding fragments of desmoplakin reveal a unique repeat motif structure. *Nature structural biology* 2002; **9**: 612-20.

81 Yin T, Green KJ. Regulation of desmosome assembly and adhesion. *Seminars in cell & developmental biology* 2004; **15**: 665-77.

82 Cabral RM, Wan H, Cole CL *et al.* Identification and characterization of DSPIa, a novel isoform of human desmoplakin. *Cell and tissue research*; **341**: 121-9.

83 Bolling MC, Jonkman MF. Skin and heart: une liaison dangereuse. *Experimental dermatology* 2009; **18**: 658-68.

84 Angst BD, Nilles LA, Green KJ. Desmoplakin II expression is not restricted to stratified epithelia. *Journal of cell science* 1990; **97 (Pt2)**: 247-57.

85 Bornslaeger EA, Corcoran CM, Stappenbeck TS *et al.* Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. *The Journal of cell biology* 1996; **134**: 985-1001.

86 Gallicano GI, Kouklis P, Bauer C *et al.* Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *The Journal of cell biology* 1998; **143**: 2009-22.

87 Gloushankova NA, Wakatsuki T, Troyanovsky RB *et al.* Continual assembly of desmosomes within stable intercellular contacts of epithelial A-431 cells. *Cell and tissue research* 2003; **314**: 399-410.

88 Yoon KH, Yoon M, Moir RD *et al.* Insights into the dynamic properties of keratin intermediate filaments in living epithelial cells. *The Journal of cell biology* 2001; **153**: 503-16.

89 Bhol K, Natarajan K, Nagarwalla N *et al.* Correlation of peptide specificity and IgG subclass with pathogenic and nonpathogenic autoantibodies in pemphigus vulgaris: a model for autoimmunity. *Proceedings of the National Academy of Sciences of the United States of America* 1995; **92**: 5239-43.

90 Spaeth S, Riechers R, Borradori L *et al.* IgG, IgA and IgE autoantibodies against the ectodomain of desmoglein 3 in active pemphigus vulgaris. *The British journal of dermatology* 2001; **144**: 1183-8.

91 Kricheli D, David M, Frusic-Zlotkin M *et al.* The distribution of pemphigus vulgaris-IgG subclasses and their reactivity with desmoglein 3 and 1 in pemphigus patients and their first-degree relatives. *The British journal of dermatology* 2000; **143**: 337-42.

92 Brandsen R, Frusic-Zlotkin M, Lyubimov H *et al.* Circulating pemphigus IgG in families of patients with pemphigus: comparison of indirect immunofluorescence, direct immunofluorescence, and immunoblotting. *Journal of the American Academy of Dermatology* 1997; **36**: 44-52.

93 Amagai. Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *J. Clin. Invest* 1994; **94**: 9.

94 Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *The Journal of clinical investigation* 1984; **74**: 313-20.

95 Stanley JR, Koulu L, Klaus-Kovtun V *et al.* A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 1986; **136**: 1227-30.

96 Amagai M, Karpati S, Prussick R *et al.* Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic. *The Journal of clinical investigation* 1992; **90**: 919-26.

97 Schiltz JR, Michel B. Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. *The Journal of investigative dermatology* 1976; **67**: 254-60.

98 Swanson DL, Dahl MV. Methylprednisolone inhibits pemphigus acantholysis in skin cultures. *The Journal of investigative dermatology* 1983; **81**: 258-60.

99 Anhalt GJ, Labib RS, Voorhees JJ *et al.* Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *The New England journal of medicine* 1982; **306**: 1189-96.

100 Amagai M, Karpati S, Klaus-Kovtun V *et al.* Extracellular domain of pemphigus vulgaris antigen (desmoglein 3) mediates weak homophilic adhesion. *The Journal of investigative dermatology* 1994; **102**: 402-8.

101 Amagai M, Ishii K, Hashimoto T *et al.* Conformational epitopes of pemphigus antigens (Dsg1 and Dsg3) are calcium dependent and glycosylation independent. *The Journal of investigative dermatology* 1995; **105**: 243-7.

102 Koch PJ, Mahoney MG, Ishikawa H *et al.* Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *The Journal of cell biology* 1997; **137**: 1091-102.

103 van der Wier G, Pas HH, Jonkman MF. Experimental human cell and tissue models of pemphigus. *Dermatol Res Pract*; **2010**: 143871.

104 Amagai M. Desmoglein as a target in autoimmunity and infection. *Journal of the American Academy of Dermatology* 2003; **48**: 244-52.

105 Caldelari R, de Bruin A, Baumann D *et al.* A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris. *The Journal of cell biology* 2001; **153**: 823-34.

106 Calkins CC, Setzer SV, Jennings JM *et al.* Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. *The Journal of biological chemistry* 2006; **281**: 7623-34.

107 Berkowitz P, Chua M, Liu Z *et al.* Autoantibodies in the autoimmune disease pemphigus foliaceus induce blistering via p38 mitogen-activated protein kinase-dependent signaling in the skin. *The American journal of pathology* 2008; **173**: 1628-36.

108 Berkowitz P, Diaz LA, Hall RP *et al.* Induction of p38MAPK and HSP27 phosphorylation in pemphigus patient skin. *The Journal of investigative dermatology* 2008; **128**: 738-40.

109 Berkowitz P, Hu P, Warren S *et al.* p38MAPK inhibition prevents disease in pemphigus vulgaris mice. *Proceedings of the National Academy of Sciences of the United States of America* 2006; **103**: 12855-60.

110 Kawasaki Y, Aoyama Y, Tsunoda K *et al.* Pathogenic monoclonal antibody against desmoglein 3 augments desmoglein 3 and p38 MAPK phosphorylation in human squamous carcinoma cell line. *Autoimmunity* 2006; **39**: 587-90.

111 Waschke J, Spindler V, Bruggeman P *et al.* Inhibition of Rho A activity causes pemphigus skin blistering. *The Journal of cell biology* 2006; **175**: 721-7.

112 Williamson L, Raess NA, Caldelari R *et al.* Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin. *The EMBO journal* 2006; **25**: 3298-309.

113 Williamson L, Hunziker T, Suter MM *et al.* Nuclear c-Myc: a molecular marker for early stage pemphigus vulgaris. *The Journal of investigative dermatology* 2007; **127**: 1549-55.

114 Muller EJ, Williamson L, Kolly C *et al.* Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *The Journal of investigative dermatology* 2008; **128**: 501-16.

115 Mao X, Payne AS. Seeking approval: present and future therapies for pemphigus vulgaris. *Curr Opin Investig Drugs* 2008; **9**: 497-504.

116 Sato M, Aoyama Y, Kitajima Y. Assembly pathway of desmoglein 3 to desmosomes and its perturbation by pemphigus vulgaris-IgG in cultured keratinocytes, as revealed by time-lapsed labeling immunoelectron microscopy. *Laboratory investigation; a journal of technical methods and pathology* 2000; **80**: 1583-92.

117 Cirillo N, Gombos F, Lanza A. Changes in desmoglein 1 expression and subcellular localization in cultured keratinocytes subjected to anti-desmoglein 1 pemphigus autoimmunity. *Journal of cellular physiology* 2007; **210**: 411-6.

118 Delva E, Jennings JM, Calkins CC *et al.* Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism. *The Journal of biological chemistry* 2008; **283**: 18303-13.

119 Mao X, Choi EJ, Payne AS. Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies. *The Journal of investigative dermatology* 2009; **129**: 908-18.

120 Hertl M, Riechers R. Analysis of the T cells that are potentially involved in autoantibody production in pemphigus vulgaris. *The Journal of dermatology* 1999; **26**: 748-52.

121 Hertl M, Amagai M, Sundaram H *et al.* Recognition of desmoglein 3 by autoreactive T cells in pemphigus vulgaris patients and normals. *The Journal of investigative dermatology* 1998; **110**: 62-6.

122 Veldman C, Stauber A, Wassmuth R *et al.* Dichotomy of autoreactive Th1 and Th2 cell responses to desmoglein 3 in patients with pemphigus vulgaris (PV) and healthy carriers of PV-associated HLA class II alleles. *J Immunol* 2003; **170**: 635-42.

123 Burge SM, Wilson CL, Dean D *et al.* An immunohistological study of desmosomal components in pemphigus. *The British journal of dermatology* 1993; **128**: 363-70.

124 Amagai M. Autoimmunity against desmosomal cadherins in pemphigus. *Journal of dermatological science* 1999; **20**: 92-102.

125 Bystryn JC, Grando SA. A novel explanation for acantholysis in pemphigus vulgaris: the basal cell shrinkage hypothesis. *Journal of the American Academy of Dermatology* 2006; **54**: 513-6.

126 Mutasim DF. Autoimmune bullous diseases: diagnosis and management. *Dermatology nursing / Dermatology Nurses' Association* 1999; **11**: 15-21; quiz 2-3.

127 Nakaji S. Current topics on immunoadsorption therapy. *Ther Apher* 2001; **5**: 301-5.

128 Grillo-Lopez AJ. Rituximab: an insider's historical perspective. *Seminars in oncology* 2000; **27**: 9-16.

129 Eisenberg R. Update on rituximab. *Annals of the rheumatic diseases* 2005; **64 Suppl 4**: iv55-7.

130 Scheinfeld N. A review of rituximab in cutaneous medicine. *Dermatology online journal* 2006; **12**: 3.

131 Reff ME, Carner K, Chambers KS *et al.* Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 1994; **83**: 435-45.

132 Taylor RP, Lindorfer MA. Drug insight: the mechanism of action of rituximab in autoimmune disease--the immune complex decoy hypothesis. *Nature clinical practice* 2007; **3**: 86-95.

133 Cardarelli PM, Quinn M, Buckman D *et al.* Binding to CD20 by anti-B1 antibody or F(ab') (2) is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol Immunother* 2002; **51**: 15-24.

134 Pedersen IM, Buhl AM, Klausen P *et al.* The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein-kinase-dependent mechanism. *Blood* 2002; **99**: 1314-9.

135 Golay J, Zaffaroni L, Vaccari T *et al.* Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 2000; **95**: 3900-8.

136 Lefebvre ML, Krause SW, Salcedo M *et al.* Ex vivo-activated human macrophages kill chronic lymphocytic leukemia cells in the presence of rituximab: mechanism of antibody-dependent cellular cytotoxicity and impact of human serum. *J Immunother* 2006; **29**: 388-97.

137 Arin MJ, Engert A, Krieg T *et al.* Anti-CD20 monoclonal antibody (rituximab) in the treatment of pemphigus. *The British journal of dermatology* 2005; **153**: 620-5.

138 Schmidt E, Hunzelmann N, Zillikens D *et al.* Rituximab in refractory autoimmune bullous diseases. *Clinical and experimental dermatology* 2006; **31**: 503-8.

139 Marzano AV, Fanoni D, Venegoni L *et al.* Treatment of refractory pemphigus with the anti-CD20 monoclonal antibody (rituximab). *Dermatology (Basel, Switzerland)* 2007; **214**: 310-8.

140 Espana A, Fernandez-Galar M, Lloret P *et al.* Long-term complete remission of severe pemphigus vulgaris with monoclonal anti-CD20 antibody therapy and immunophenotype correlations. *Journal of the American Academy of Dermatology* 2004; **50**: 974-6.

141 Ahmed AR, Spigelman Z, Cavacini LA *et al.* Treatment of pemphigus vulgaris with rituximab and intravenous immune globulin. *The New England journal of medicine* 2006; **355**: 1772-9.

142 Joly P, Mouquet H, Roujeau JC *et al.* A single cycle of rituximab for the treatment of severe pemphigus. *The New England journal of medicine* 2007; **357**: 545-52.

143 Eming R, Nagel A, Wolff-Franke S *et al.* Rituximab exerts a dual effect in pemphigus vulgaris. *The Journal of investigative dermatology* 2008; **128**: 2850-8.

144 Keystone E, Fleischmann R, Emery P *et al.* Safety and efficacy of additional courses of rituximab in patients with active rheumatoid arthritis: an open-label extension analysis. *Arthritis and rheumatism* 2007; **56**: 3896-908.

145 Collins-Burow B, Santos ES. Rituximab and its role as maintenance therapy in non-Hodgkin lymphoma. *Expert review of anticancer therapy* 2007; **7**: 257-73.

146 Niedermeier A, Eming R, Pfutze M *et al.* Clinical response of severe mechanobullous epidermolysis bullosa acquisita to combined treatment with immunoadsorption and rituximab (anti-CD20 monoclonal antibodies). *Archives of dermatology* 2007; **143**: 192-8.

147 Amagai M. Pemphigus as a paradigm of autoimmunity and cell adhesion. *The Keio journal of medicine* 2002; **51**: 133-9.

148 Suter MM, Wilkinson JE, Dougherty EP *et al.* Ultrastructural localization of pemphigus vulgaris antigen on canine keratinocytes in vivo and in vitro. *American journal of veterinary research* 1990; **51**: 507-11.

Chapter 2

Pemphigus patient skin: IgG induced selective relocation of desmoglein explains the granular IgG deposition pattern

D.A.M. Oktarina[1], G. van der Wier[1], G.F.H. Diercks, M.F. Jonkman and H.H. Pas

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

Submitted

1. The first two authors contributed equally.

Abstract

Background. In pemphigus circulating IgG is present to the desmosomal adhesion molecules desmoglein (Dsg) 1 and 3. In the epidermis of patients, this IgG deposits in an uneven granular pattern not reflecting the normal Dsg distribution.
Objectives. To understand why the IgG deposits in a granular pattern in pemphigus patient skin.
Patients/Methods. We analyzed the distribution of IgG and desmosomal adhesion molecules in skin biopsies of 18 pemphigus vulgaris (PV) and 10 pemphigus foliaceus (PF) patients by double staining immunofluorescence. The effect of IgG on desmosomal proteins was studied in an *in vitro* skin model.
Results. In PF skin Dsg1, but not Dsg3, had lost its normal expression pattern and was present in the same granular pattern as the IgG deposits. Vice versa, in skin of PV patients with anti-Dsg3 antibodies Dsg3, but not Dsg1, colocalized with the granular IgG deposits. Also plakoglobin (PG) had reallocated and co-concentrated with IgG and Dsg, but this was far more prominent in skin of patients with anti-Dsg1 antibodies than in skin of patients with anti-Dsg3 antibodies. In areas of heavy Dsg1 aggregation, but not in areas of heavy Dsg3 aggregation, widening between cells was present. Patient IgG, but not patient Fab fragments, induced the same Dsg reallocations in normal human skin *in vitro.*
Conclusions. The IgG induced rearrangement of the Dsg autoantigens is responsible for the granular IgG deposition pattern in patient skin. In PF and in mucocutaneous PV skin Dsg1 aggregation but not Dsg3 aggregation correlates with non-acantholytic intercellular widening.

Introduction

Pemphigus is an autoimmune blistering disease characterized by intraepidermal deposition of IgG and blistering of the skin and/or mucous membranes. The two major types of pemphigus comprise pemphigus vulgaris (PV), always affecting mucosa and in approximately half of the cases skin, and pemphigus foliaceus (PF) that affects skin only. In PV IgG against desmoglein 3 (Dsg3) is held responsible for suprabasal acantholysis while in PF IgG against Dsg1 provokes subcorneal acantholysis [1-4].

Desmogleins, belonging to the cadherin family, are desmosomal proteins that link keratinocytes. Two opposing desmogleins bind each other with the extracellular N-terminal domains. Their cytoplasmic domains are indirectly connected to the keratin filaments through desmoplakin (DP) that binds intermediate filaments, and plakoglobin (PG) that links desmoplakin to the cytoplasmic tails of the cadherins. Dsg3 is more abundantly present in the lower epidermis but absent in the subcorneal layer, while Dsg1 expression increases from the basal to the subcorneal layer. This differential distribution is the basis of the desmoglein compensation hypothesis that explains the difference in separation levels between PV and PF by Dsg isoform redundancy [5,6].

The mechanism by which IgG induces acantholysis remains a matter of debate. Four concepts prevail today. 1) Binding of IgG to Dsg disrupts homophilic transinteraction, leading to lengthwise splitting of desmosomes and loss of cell-cell adhesion [7]. 2) Binding of IgG to Dsg derails intracellular signalling pathways ending with cytoskeleton collapse or desmosome disassembly [8-12]. 3) Binding of IgG induces loss of Dsg3 through endocytosis what leads Dsg3-depleted desmosomes with decreased adhesive strength [13] or 4) to severe assembly problems and inflation wasting of desmosomes [14]. For all concepts supportive evidence is present in the literature what underlines the vast complexity of investigating the pathogenic process of acantholysis.

Most of today's concepts originate from experimental models and relatively little attention has been given to patient skin. Those who perform diagnostic immunofluorescence microscopy (IF) know that there is a strange discrepancy between *in vivo* IgG deposition by direct IF on patient skin and the intercellular substance (ICS) pattern by indirect IF that is described to be typical for pemphigus. The ICS pattern is a smooth staining around the epidermal cells, also called honeycomb or chicken wire pattern, and is in line with the normal distribution of the Dsg's. In patient skin however the IgG deposits have a granular appearance [15]. In the present study we investigated multiple patient skin biopsies in order to understand why the IgG deposits *in vivo* in such an aberrant granular fashion and if that granular deposition is connected with morphologic changes of the skin cells.

Materials and methods

Patient samples

Forty biopsies of 28 pemphigus patients: 3 with mucosal PV, 15 with mucocutaneous PV

and 10 with PF were included for immunofluorescence. All biopsies had been immediately frozen in liquid nitrogen and stored at -80°C. All diagnoses had been established on basis of clinical criteria and laboratory data, including histology and immunofluorescence. Their anti-Dsg antibody profiles were determined by Dsg1 and Dsg3 enzyme-linked immunosorbent assays (ELISA) (MBL, Nagoya, Japan). The site of biopsy included lesional and/or non-lesional skin. Skin was considered non-lesional if it seemed healthy at physical examination and Nikolsky's sign was negative. Biopsies from redundant breast reduction skin served as healthy controls. Electron microscopy was performed for two glutaraldehyde-fixed biopsies, from Nikolsky-negative skin of a mucosal PV patient and from Nikolsky-positive skin of a PF patient. For the *in vitro* studies four PF sera and four mucocutaneous PV sera were used.

Purification of IgG and generation of Fab fragments

IgG was purified from sera by protein G sepharose chromatography (HiTrap Protein G HP, GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocols. The purified IgG was divided into two equal fractions. One fraction was dialyzed against PBS (10 mM sodium phosphate, 150 mM NaCl pH 7.2), concentrated by ultrafiltration (Amicon Ultra 100K NMWL, Millipore, Co Cork, Ireland) to 0.5 ml and mixed with 3 ml DMEM/HAM 1:3 medium containing 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptromycin. After a final concentration step the solution was stored at -80°C. From the second fraction Fab fragments were prepared using immobilized papain (Pierce, Rockford, USA) according to the manufacturer's protocol. The Fab fragments were separated from the undigested IgG and the Fc-tail by protein A sepharose chromatography (HiTrap Protein A HP, GE Healthcare, Uppsala, Sweden). The flow-through containing the Fab fragments was concentrated (Amicon Ultra 30K NMWL, Millipore, Co Cork, Ireland) to 0.5 ml, mixed with 3 ml of the same medium as used for the IgG, concentrated again and stored at -80°C.

In vitro model

Six millimeter biopsies from redundant breast reduction skin were placed on transwell inserts in a 24-wells plate (Corning, New York, USA). The IgG and Fab fractions were added to the wells, such that the medium contacted the bottom of the insert, and the plate was incubated at 37 °C under cell-culture conditions. After 24 hours the biopsies were taken out, briefly rinsed with HBSS (Gibco, Glasgow, UK), frozen in liquid nitrogen and stored at -80 °C.

Immunofluorescence microscopy

The procedure for immunofluorescence staining and image collection has been described before in detail [16]. For visualization of adhesion molecules we used the following monoclonals: Dsg1-P23 and 27B2 (Dsg1), 10G11 (Dsg2), Dsg3-G194 (Dsg3), U100 (desmocollin (Dsc) 1), U114 (Dsc3), 15F11 (PG), DP2.15 (DP), PKP3-270.6.2 (plakophilin (PKP) 3), NCH-38 (E-cadherin), 9G2 (β-catenin) and HD121 (plectin). Double staining of IgG and adhesion molecules IgG was performed with fluorescein-conjugated Fcγ-specific goat F(ab')2

anti-human IgG (Protos Immunoresearch, Burlingame, CA, U.S.A) and Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes Eugene, OR, U.S.A) as secondary steps. For visualizing Fab fragments, we used a fluorescein-conjugated rabbit anti-human light chain kappa and lambda Ig (Dako Cytomation, Denmark). For double staining with two different mouse monoclonals we used Zenon® Mouse IgG Labeling Kits Alexa Fluor®488 and Alexa Fluor®568 (Molecular Probes, Invitrogen, USA) by following technical protocols from the company.

Electron microscopy

The biopsies were fixed in 2% glutaraldehyde and postfixed with 1% osmium tetroxide and 1,5% potassium ferrocyanide in 0.1 M sodium cacodylate buffer. After dehydration in alcohol they were embedded in epon and ultrathin sections were cut. These were stained with uranyl acetate and lead citrate and examined with a Philips CM100 transmission electron microscope.

Results

All investigated biopsies had intraepidermal deposition of IgG. This IgG did not have the smooth ICS distribution that is seen in indirect immunofluorescence when pemphigus serum is brought on a section of normal skin (Figure S5). Instead, in most biopsies an uneven deposition was present giving the overall pattern an irregular, somewhat speckled, appearance. Sometimes the IgG was present as interrupted lines but more often it had concentrated in a limited number of fine or coarse clusters per individual cell. We next investigated the distribution of adhesion molecules in these biopsies.

Aggregation of IgG, Dsg3 and PG in non-lesional skin of patients with IgG to Dsg3 only

In normal human skin Dsg3 is present with a declining expression from lower to higher layers and absent in the subcorneal layers, while reverse Dsg1 increases upwards from the basal layer and is present throughout the whole epidermis (Figure 1 a-c). In patient skin Dsg3 had lost this normal distribution and was present as dots with Dsg1 still present in the normal pattern (Figure 1 d-f). Desmocollin (Dsc) 3 (Figure 1 g-j) and Dsc1 (not shown) also remained normally distributed. The overall Dsg3 fluorescence intensity was weak compared to normal human skin. As expected the deposited IgG co-localized with the dotted Dsg3 (Figure 1 j-l). The intracellular plaque proteins DP, plakophilin 3 (PKP3) and plectin remained smoothly distributed (Figure S1 a-i). PG was largely normal but some slight concentrations were visible at the positions of the Dsg3 dots (Figure 1 m-o). Proteins of the adherens junction as beta-catenin (B-cat) did not demonstrate any shifts in localization (Figure S1 j-l). The IgG deposition pattern can thus be explained by an altered distribution of the Dsg3 antigen to which the IgG binds. As all other cadherins and desmosomal plaque proteins do not concentrate in the Dsg3 dots, the plausible explanation is that desmosomes become depleted of

Dsg3.

Aggregation of IgG and desmosomal proteins in skin of PF patients

In PF, both in lesional and in non-lesional skin, the IgG-deposits were granular in the lowers layers and most times more smooth in the higher layers, although in incidental biopsies the IgG had an almost complete granular appearance (Figure S2 b and c). When a blister was present the granular deposits were most often located underneath it, although in some biopsies they were also seen above the blisters (Figure S2 d and e). A few biopsies had little IgG deposition and here local concentrations of IgG were present in the lower half of the

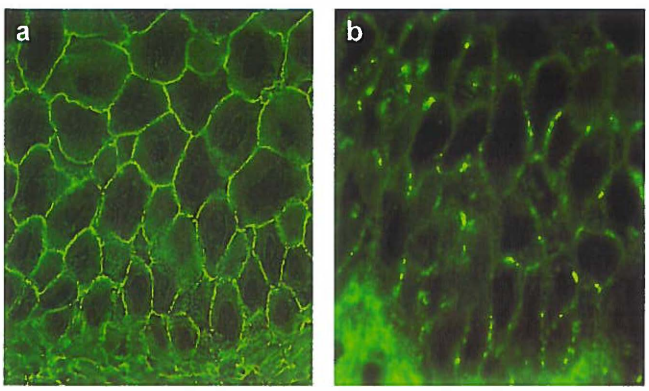


Figure S5. Dotted pattern of IgG deposition in PV skin. When PV serum is brought on a section of normal healthy epithelium, here monkey esophagus, the IgG binds in a smooth pattern around the keratinocytes (**a**). The *in vivo* IgG deposition pattern in the skin of the same patient is totally different, and demonstrates a dotted pattern that seems to be located along the cell membranes (**b**).

basal cells close to the basement membrane (Figure S2 a). Compared to mucosal PV skin the aggregates had a more coarse appearance. Both Dsg1 and PG co-localized with the IgG. In contrast to mucosal PV skin the distribution of PG was much more distorted. Where in PV most of the PG kept its smooth membrane distribution, in PF skin it was far more aggregated such that a major part of the PG had concentrated in these dots (Figure 2 a-c). The other cadherins Dsc1, Dsc3 and Dsg3 did not demonstrate this aggregation (Figure 2 d-l). In areas of heavy PG clustering also some concentration of other plaque molecules was visible. Figure 2 (m-r) shows that that DP and PKP3 to some extent follow their binding partner PG. Adherens junction proteins E-cadherin and beta-catenin did not aggregate (Figure S1 m-r).

Aggregation patterns in skin of patients with IgG to Dsg1 and to Dsg3.

As expected both types of aggregates were present in healthy skin of mucocutaneous PV patients whose IgG targets both Dsg's. Although we found some aggregates to be dominantly of the IgG/Dsg3/PG type and some of the IgG/Dsg1/PG type they most times seemed to have concentrated at the same positions. Between patients the degree of clustering of the individual Dsg's could widely differ. Patients with relatively high anti-Dsg1 ELISA values demonstrated heavier Dsg1 clustering whereas anti-Dsg3 clustering dominated in patients

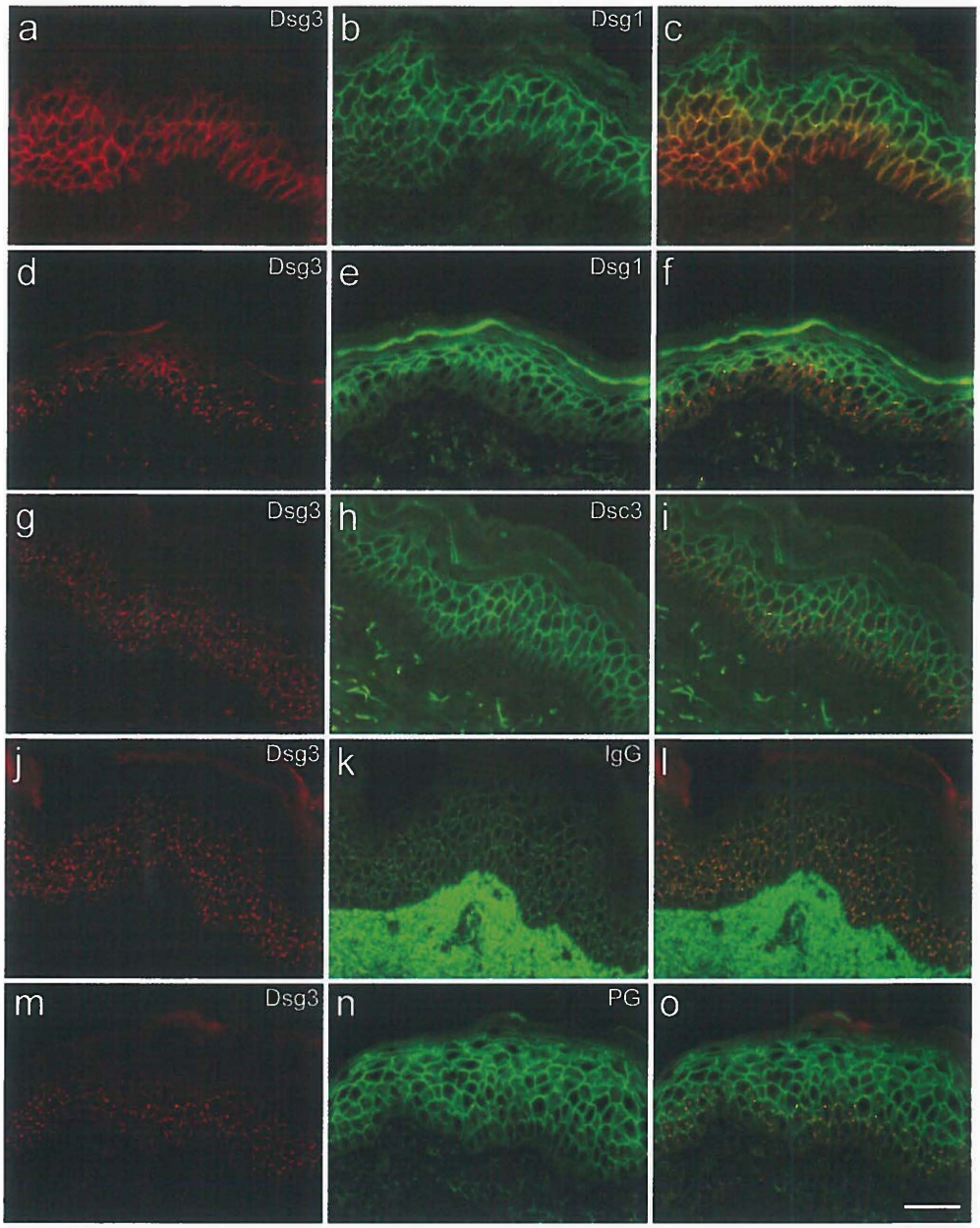
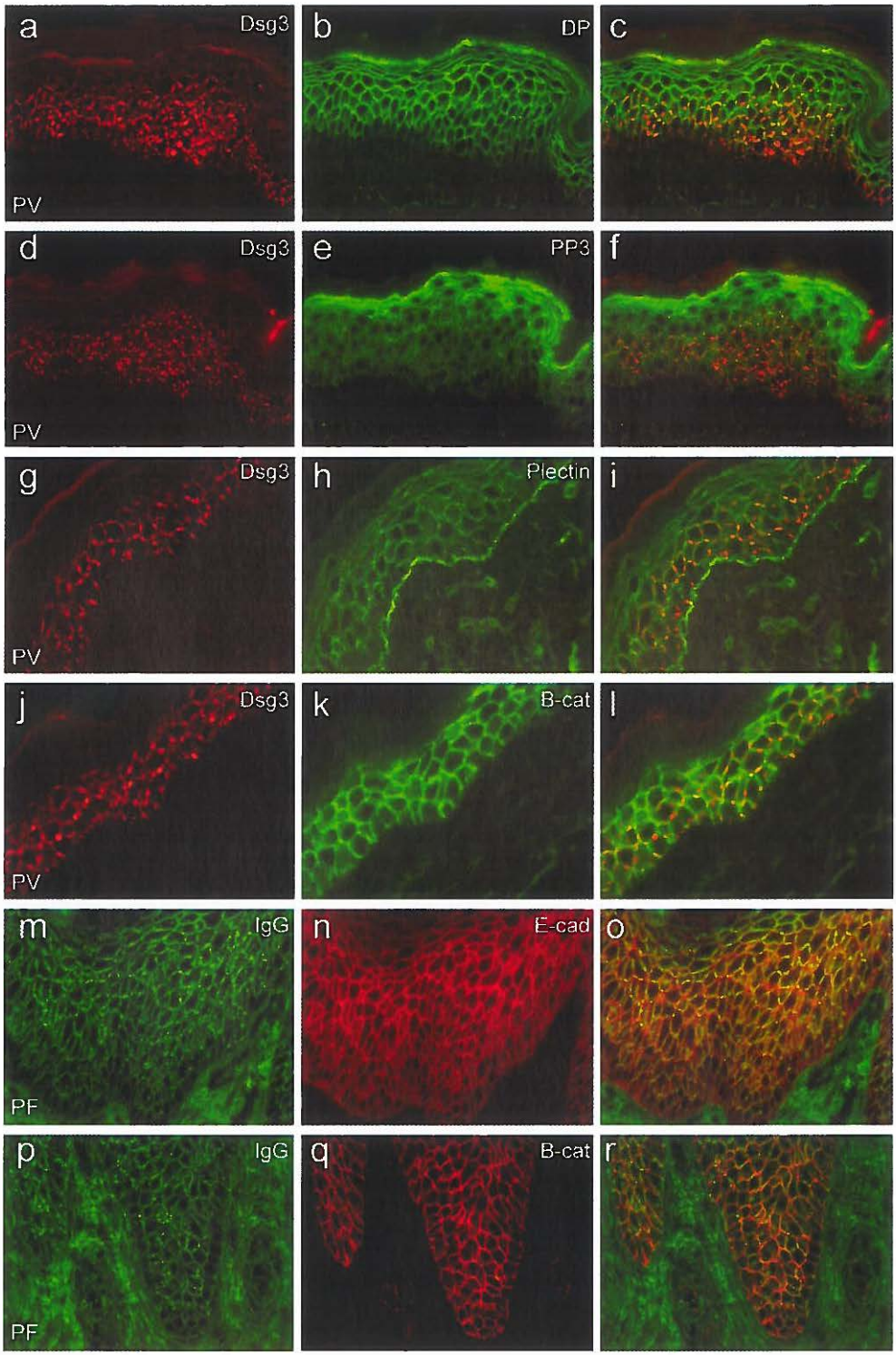


Figure 1. Distribution of IgG and desmosomal adhesion molecules in healthy skin of PV patients with IgG antibodies to desmoglein 3. Double immunofluorescence staining (**c, f, i, l,** and **o** are overlays from the reds and greens left of them) shows a dotted epidermal distribution of desmoglein 3 (Dsg3) in PV patient skin (**d, g, j, m**) that contrasts with the Dsg3 distribution in normal human skin (**a**). The Dsg3 dots colocalize with the IgG (**k**) that is deposited in the same PV skin. Other cadherins as Dsg1 (**e**) do not concentrate in these dots and remain distributed as in normal human skin (**b**). Also Dsc3 (h) does not follow the Dsg3. Some plakoglobin (PG) (**n**) colocalizes with the Dsg3 dots. All images have the same magnification. The white bar is 40 micrometers.


a
Dsg3
PV
b
DP
c
d
Dsg3
PV
e
PP3
f
g
Dsg3
PV
h
Plectin
i
j
Dsg3
PV
k
B-cat
l
m
IgG
PF
n
E-cad
o
p
IgG
PF
q
B-cat
r

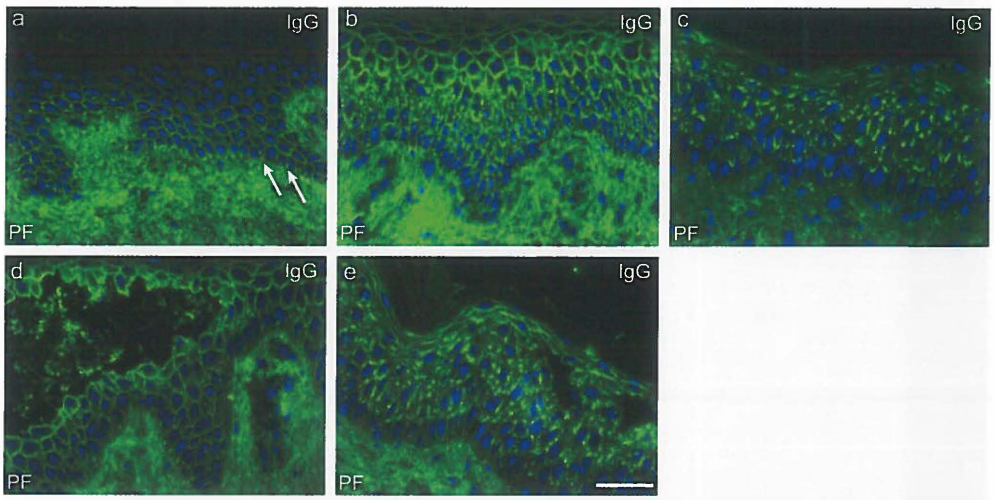


Figure S2. IgG deposition patterns in PF patient skin. Immunofluorescence demonstrated different IgG deposition patterns in PF patient skin. In skin with little IgG deposition (**a**) granular aggregation was already present but close to the BMZ (white arrows). Other samples showed more intense IgG deposition that could be granular in lower cell layers and smoother in higher cell layers (**b** and **d**) or granular all over (**c** and **e**). This was observed in both non-lesional (**b** and **c**) and lesional skin (**d** and **e**).

Figure S1. (Opposite page) Distribution of desmosomal plaque and adherens junction molecules in healthy skin of PV and PF patients. In PV skin no colocalization with the Dsg3 dots (**a, d, g,** and **j**) is seen for other desmosomal plaque molecules proteins desmoplakin (**b**), plakophilin-3 (**e**), plectin (**h**) or with adherens junction molecules as shown here for beta-catenin (**k**). Also in PF skin the adherens junctions molecules E-cadherin (**n**) and beta-catenin (**q**) do not colocalize with the dots represented here by the deposited IgG (**m** and **p**). Images **c, f, i, l, o** and **r** are overlays from the reds and greens left of them.

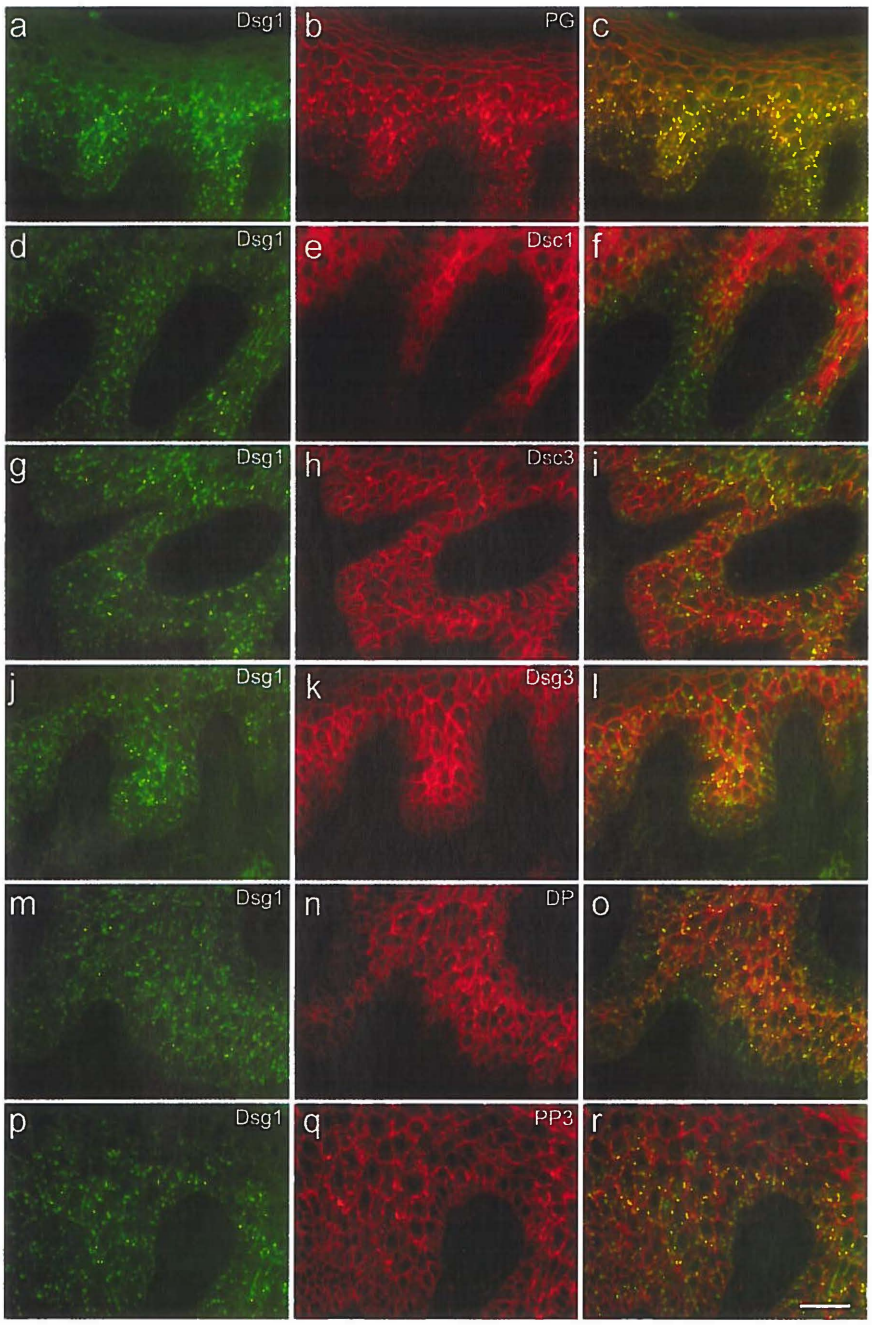


Figure 2. Distribution of desmosomal adhesion and plaque molecules in skin of PF patients. Double immunofluorescence staining of PF patient skin reveals a dotted epidermal distribution of Dsg1 (**a, d, g, j, m** and **n**). The distribution of the plaque molecule PG (**b**) follows that of Dsg1 and is present in the same dots. The other cadherins Dsc1 (**e**), Dsc3 (**h**) and Dsg3 (**k**) do not follow the rearranged Dsg1. The plaque proteins desmoplakin (**n**) and plakophilin-3 (**q**) partly concentrate at the same locations as PG and Dsg. Images **c, f, i, l, o** and **r** are overlays from the reds and greens left of them. All images have the same magnification. The white bar is 40 micrometers.

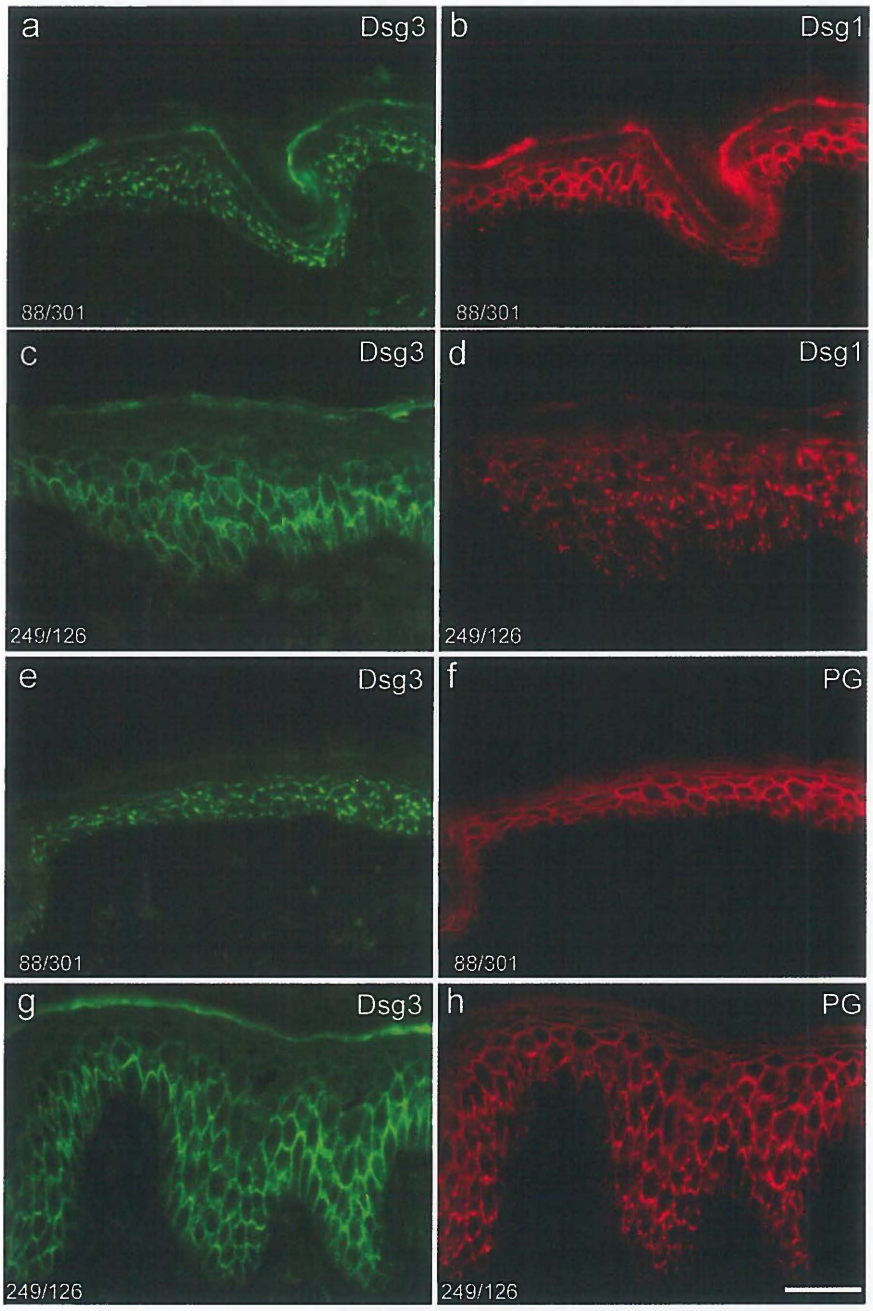


Figure 3. Distribution of Dsg1, Dsg3 and PG in skin of patients with IgG to both Dsg1 and Dsg3. Both Dsg1 (**b, d**) and Dsg3 (**a, c, e, g**) become rearranged. The extent to which individual cadherins become concentrated in dots differs between patients and seems to correlate with their respective anti-Dsg titres. Images **a, b** and **e, f** are from a patient with ELISA index values of 249 for Dsg1 and 126 for Dsg3 and images **c,d** and **g,h** from a patient with index values of respectively 88 and 301. PG (**f, h**) did not follow the distribution of Dsg3 (**e, g**), but aggregated with similar intensity as Dsg1 (compare image **b** with **f** and **d** with **h**). All images were obtained trough double staining and were photographed in separate channels, thus reds and greens represent the same section. The white bar is 40 micrometers.

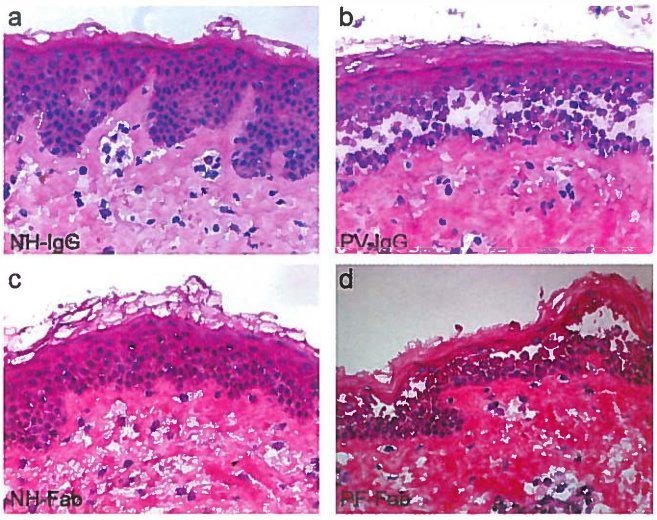


Figure S3. IgG and Fab fragments both induce the typical pemphigus blisters. Normal human skin biopsies incubated with pemphigus IgG or Fab fragments induced blistering identical to that observed in pemphigus patient skin. Shown are the typical PV suprabasal blister that was induced with PV IgG (**b**) and the subcorneal PF blister that was induced here with PF Fab fragments (**d**). Control incubations with normal human (NH) IgG (**a**) or normal human Fab fragments (**c**) did not show blistering or acantholysis.

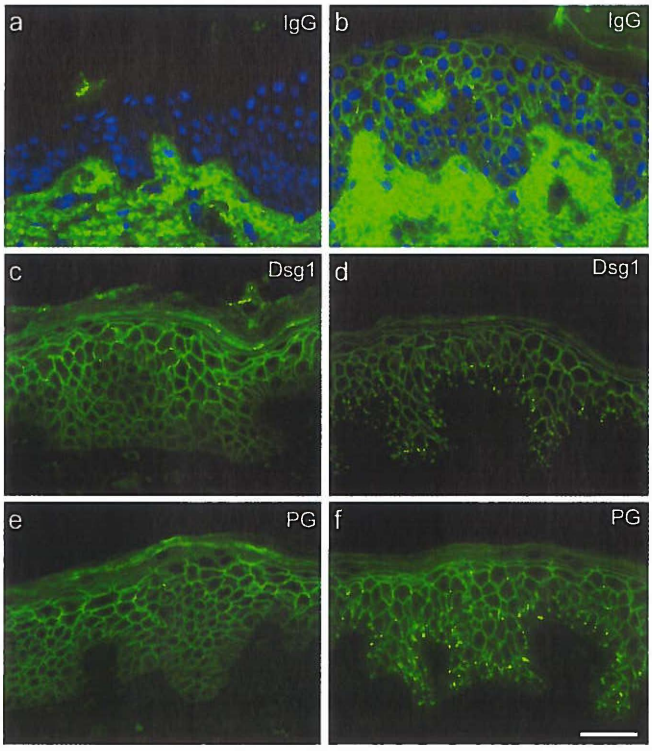


Figure S4. The rearrangement of Dsg and PG into clusters is induced by pemphigus patient IgG. When biopsies of normal human skin were incubated with PF IgG (**b, d, f**) the IgG (**b**) became bound in the epidermis in a similar granular pattern as observed in patient skin. Both Dsg1 (**d**) and PG (**f**) rearranged into these same clusters. When incubated with normal human IgG (**a, c, e**) the IgG (**a**) became not bound in the epidermis and redistribution of Dsg1 (**c**) or PG (**e**) did not occur. All images have the same magnification. The white bar is 40 micrometers.

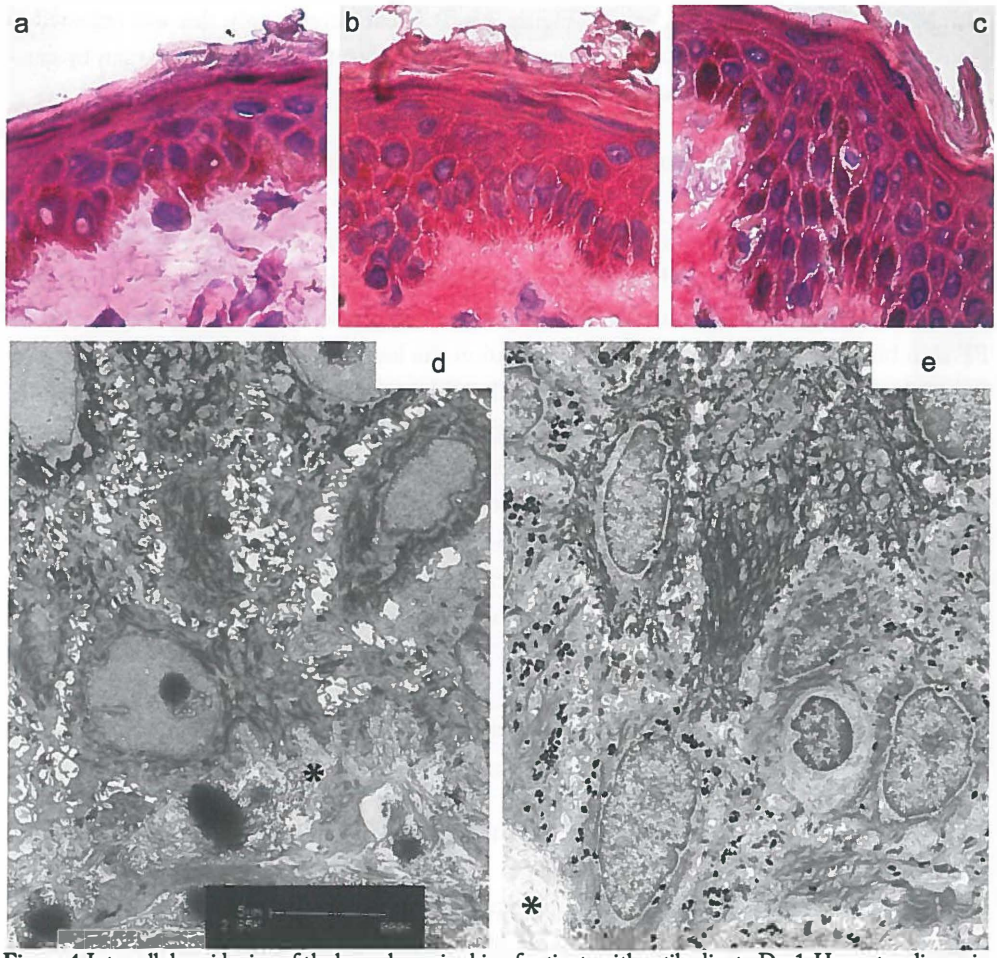


Figure 4. Intercellular widening of the lower layers in skin of patients with antibodies to Dsg1. Haematoxylin-eosin stained sections of non-lesional skin of a mucosal PV patient with IgG to Dsg3 only (**a**), of non-lesional skin of a PF patient (**b**) and of non-lesional skin of a mucocutaneous PV patient with IgG antibodies to both Dsg1 and Dsg3 (**c**). Subtle intercellular widening of the intercellular space is seen in the skin of the PF and the mucocutaneous PV patient, but not in the skin of the patient with mucosal PV. Electron microscopy demonstrates intercellular widening in Nikolsky-positive non-lesional PF skin (**d**) but not in non-lesional mucosal PV skin (**e**). The asterisk denotes the dermal compartment.

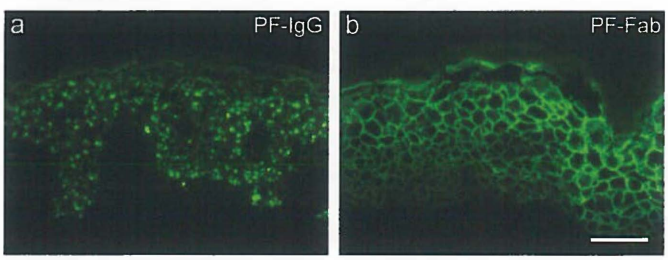


Figure 5. IgG but not Fab fragments induce clustering. In skin that was incubated with PF IgG the PG aggregated (**a**), while in contrast in skin that was incubated with Fab fragments prepared from the same IgG the PG did not become affected (**b**). Nevertheless the Fab fragments induced blistering at the subcorneal level. Both images have the same magnification. The white bar is 40 micrometers.

with higher anti-Dsg3 ELISA values (Figure 3 a-d). Most interestingly, this was reflected in the degree of PG aggregation. PG was severely more reallocated by anti-Dsg1 than by anti-Dsg3 clustering (Figure 3 e-f shows Dsg3 versus PG).

Intercellular widening of the lower layers in non-lesional PF and mucocutaneous PV skin

To investigate if aggregation of Dsg1 or of Dsg3 leads to an altered cellular morphology we investigated the histology of the biopsies used for immunofluorescence. The skin of patients with only aggregated Dsg3 did not show any abnormalities on HE staining (Figure 4a). In PF skin biopsies with profound Dsg1 aggregation the keratinocytes exhibited subtle widening of the intercellular space especially of the basal layer and to a lesser degree of the suprabasal layer (Figure 4b). In lesional skin also higher layers widened. The widening roughly correlated with the degree of Dsg1 clustering. Biopsies without visible widening had little IgG deposition and minimal Dsg1 clustering. Widening was also present in uninvolved skin of mucocutaneous PV patients who had additional antibodies to Dsg1, and whose skin demonstrated substantial PG aggregation (Figure 4c). As intercellular widening of the lower layers had been described before in electron microscopic studies of both PV and PF skin [17,18] we also performed electron microscopy on a biopsy of Nikolsky-positive PF skin, and on a biopsy of mucosal PV skin of which the corresponding IF freeze biopsy demonstrated Dsg3 aggregation. Electron microscopy showed intercellular widening of the basal layer in the PF biopsy but not in the mucosal PV biopsy (Figure 4d and 4e).

Pemphigus IgG but not Fab fragments induce the typical aggregation patterns in skin

In the *in vitro* model the typical PV blisters could be evoked within 24 hours in normal human skin. Incubation with IgG or Fab fragments from PV patients induced suprabasal blisters while in contrast PF IgG or Fab induced the typical subcorneal blisters (Figure S3 a-d). The IgG became bound in the epidermis in the same speckled manner as in patient biopsies (Figure S4b). Likewise, the targeted Dsg clustered, and PG co-clustered with the IgG and the Dsg1 (Figure S4d and S4f). In contrast, Fab fragments bound smoothly around the cells and no aggregation was seen, neither of Dsg nor of PG (Figure 5). The aggregation of Dsg therefore results from an intrinsic characteristic of the IgG not present in the Fab fragments. The difference between IgG and Fab fragments is that the IgG is bivalent and therefore able to crosslink Dsg's, while in contrast Fab fragments are monovalent and will only bind a single Dsg molecule. Incubation with normal human IgG or Fab fragments did not show any of these effects although after 24 hours some spongiosis was present due to the incubation conditions (not shown).

Discussion

Our results show that the granular IgG deposition pattern in skin of pemphigus patients is

explained by IgG induced selective aggregation of the targeted Dsg. The *in vitro* experiments demonstrated that this is caused by cross-linking of Dsg molecules due to the bivalency of the IgG. In addition, patient skin revealed an interesting differential effect of PV and PF IgG on PG showing that disorganization of Dsg1 has much greater impact on PG than the disorganization of Dsg3. Furthermore, we put forward a new idea on intercellular widening in the lower epidermal layers of patient skin of mucocutaneous PV and PF as we hypothesize that this widening is related with anti-Dsg1 antibodies.

In the skin of patients with anti-Dsg3 antibodies Dsg3 is decreased and present in a granular pattern. Our observations confirm Shu *et al.* who demonstrated that less than normal Dsg3 could be extracted from patient skin [19]. Also in experimental cell culture models a decrease in Dsg3 is observed. Adding PV-IgG to cultured keratinocytes starts depletion of Dsg3, first of non-desmosomal Dsg3 and then gradually as no Dsg3 is incorporated anymore in newly formed desmosomes, also of desmosomal Dsg3 suggesting that the remaining desmosomes become depleted of Dsg3 [13,19-21]. Our patient skin data favour this hypothesis, as the residual Dsg3 was present in only a few clusters per cell while simultaneously other cadherins kept their normal distribution. Most important however, we observed that this disorganization and depletion takes place in skin that does not show signs of acantholysis or pre-acantholysis. Our pure mucosal PV patients had clinically healthy skin that never blistered during follow-up. At the same time these patients had blisters in the oral mucosa, demonstrating that the antibodies are capable of inducing pathogenesis. Clearly, epidermis is able to withstand the pathogenic effects of anti-Dsg3 antibodies and as such it differs from mucosal epithelium. Although cell experiments demonstrated decreased mechanical-stress resistance in Dsg3-depleted cultured monolayer sheets [13,21], the skin of our patients was functioning properly, was not susceptible to minor trauma or friction and Nikolsky's sign could not be evoked.

In PF skin Dsg1 aggregates instead of Dsg3. From the different biopsies a picture emerged of an aggregation process that starts low in the basal layer, close to the BMZ and that, when more IgG becomes available, also spreads to the layers above. That the basal layer shows the earliest changes is not surprising as that layer contacts the IgG first. Other cadherins do not reallocate and this suggests, in analogy with Dsg3-depletion in PV, that desmosomes probably become depleted of Dsg1. PF blisters never occur in the lower layers thus sufficient compensation is present there to prevent the cells from becoming acantholytic although cell-cell widening can be present as will be discussed further below.

The striking difference between PV and PF is the extent to which the PG co-clusters. Where in healthy PV skin PG largely maintains its normal distribution it becomes in PF skin heavily disturbed and is seen as large dots together with Dsg1 while only a small part remains distributed along the cell membrane. Possibly PG is more tightly bound to Dsg1 as to Dsg3. The actual strength of binding of both cadherins with PG is not known, but analysis of the stoichiometry of PG-cadherin complexes has revealed that Dsg3 binds PG in a 1:1 ratio where Dsg1 binds two molecules of PG [22]. Although this might explain the observed

difference, a second explanation involving the phosphorylation state of Dsg is as plausible. Dsg3 becomes phosphorylated when IgG binds to it and this results in dissociation of PG from Dsg3 [23,24]. Binding of IgG and subsequent redistribution of Dsg3 therefore may have little effect on PG. For Dsg1 it is currently unknown if binding of IgG results in similar phosphorylation and dissociation. Our patient skin data however suggest that this might not be the case seen the extent of colocalization of Dsg1 and PG.

The widening between cells of the lower layers correlates with aggregation of Dsg1 but not with aggregation of Dsg3. Apparently, the aggregation of Dsg1 has a more profound effect on cell-cell cohesion than the aggregation of Dsg3. This might be related to a more important role for Dsg1 itself in intercellular adhesion of the lower layers than assumed so far. Depletion of Dsg1 from desmosomes might lead to less or weaker desmosomes than depletion of Dsg3. Alternatively there may be a role for non-desmosomal Dsg1 in interdesmosomal adhesion. A third attractive explanation is that the widening is due to the redistribution of PG. PV biopsies with only little PG redistribution do not show this widening. What we did observe at the positions of the PG clusters was that other desmosomal plaque proteins as PKP3 and DP also partly concentrated at these same spots, although with less intensity as PG and Dsg1, but enough to suggest that some molecules were still bound to PG and dragged along with it. Such complexes hypothetically could still be capable of linking up to keratin filaments and become part of the dynamics of the cytoskeleton. If these complexes cannot withstand the force exerted on them by the cytoskeleton, they might be drawn inwards what could lead to local separation between adjacent cell membranes. At this moment however all hypotheses remain pure speculation.

It is without doubt however that in PF skin widening is present in layers where no acantholysis will take place. Although ultrastructural studies are limited in PF, they are in line with our observation. Wilgram *et al.* observed widening between basal cells that was described by them as 'early acantholysis' [17]. When IgG from endemic PF patients was injected in mice already after 1 to 3 hours widening of interdesmosomal areas of the basal and spinous layer occurred [25]. In this respect it is interesting that interdesmosomal widening is also seen in skin of PV patients, and after injection of PV serum into mice [18,26]. Based on these publications widening is considered an early event in PV that proceeds acantholysis, and moreover also has initiated alternate theories involving basal cell shrinkage as a cause in PV [27]. It is important however to realize that these ultrastructural studies date back to the time that the antibody specificity of patients was not known and, considering the fact that they likely were mucocutaneous patients with both anti-Dsg3 and anti-Dsg1 antibodies, it is conceivable that the early widening in PV is caused by concomitant anti-Dsg1 antibodies. A definitive answer can only be provided by repeating the electron microscopic studies with a series of defined patient material.

How does patient skin comply with current hypotheses on PV acantholysis? Seen the altered distribution and decreased Dsg3 level, we do not doubt that the selective depletion of Dsg3 from desmosomes that is repeatedly shown in cultured cell models, is also present

in patient skin. We therefore favour the Dsg3-depleted desmosome model as suggested by Aoyama *et al.* [13]. We would however like to expand this model with Dsg1-depletion based on our observation that Dsg3-depleted patient skin is sturdy and Nikolsky-negative. The compensatory mechanisms in skin evidently are of such strength that they prevent skin from becoming Nikolsky-positive. Based on our observations on PF skin, that suggests that anti-Dsg1 antibodies deplete desmosomes of Dsg1, we hypothesize that in mucocutaneous PV skin concomitant Dsg1-depletion will further weaken desmosomes leading to Nikolsky-positive and finally acantholytic skin.

Acknowledgments

This work was in part supported by the Groningen Bernoulli Fund and a Schlumberger Foundation Faculty for the Future grant to DAMO and a J.P. Naterfonds grant to GvdW.

References

1 Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67**: 869-77.

2 Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *The Journal of clinical investigation* 1984; **74**: 313-20.

3 Stanley JR, Koulu L, Klaus-Kovtun V *et al.* A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 1986; **136**: 1227-30.

4 Amagai M, Hashimoto T, Shimizu N *et al.* Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *The Journal of clinical investigation* 1994; **94**: 59-67.

5 Amagai M. Pemphigus as a paradigm of autoimmunity and cell adhesion. *The Keio journal of medicine* 2002; **51**: 133-9.

6 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *The Journal of clinical investigation* 1999; **103**: 461-8.

7 Amagai M, Karpati S, Prussick R *et al.* Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic. *The Journal of clinical investigation* 1992; **90**: 919-26.

8 Berkowitz P, Hu P, Liu Z *et al.* Desmosome signaling. Inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization. *The Journal of biological chemistry* 2005; **280**: 23778-84.

9 Nguyen VT, Arredondo J, Chernyavsky AI *et al.* Pemphigus vulgaris acantholysis ameliorated by cholinergic agonists. *Archives of dermatology* 2004; **140**: 327-34.

10 Delva E, Jennings JM, Calkins CC *et al.* Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism. *The Journal of biological chemistry* 2008; **283**: 18303-13.

11 Muller EJ, Williamson L, Kolly C *et al.* Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *The Journal of investigative dermatology* 2008; **128**: 501-16.

12 Williamson L, Hunziker T, Suter MM *et al.* Nuclear c-Myc: a molecular marker for early stage pemphigus vulgaris. *The Journal of investigative dermatology* 2007; **127**: 1549-55.

13 Aoyama Y, Nagai M, Kitajima Y. Binding of pemphigus vulgaris IgG to antigens in desmosome core domains excludes immune complexes rather than directly splitting desmosomes. *The British journal of dermatology* 2010 PMID: 20222919

14 Mao X, Choi EJ, Payne AS. Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies. *The Journal of investigative dermatology* 2009; **129**: 908-18.

15 Burge SM, Wilson CL, Dean D *et al.* An immunohistological study of desmosomal components in pemphigus. *The British journal of dermatology* 1993; **128**: 363-70.

16 Vodegel RM, Jonkman MF, Pas HH *et al.* U-serrated immunodeposition pattern differentiates type VII collagen targeting bullous diseases from other subepidermal bullous autoimmune diseases. *The British journal of dermatology* 2004; **151**: 112-8.

17 Wilgram GF, Caulfield JB, Madgic EB. An Electron Microscopic Study of Acantholysis and Dyskeratosis in Pemphigus Foliaceus: with a Special Note on Peculiar Intracytoplasmic Bodies. *The Journal of investigative dermatology* 1964; **43**: 287-99.

18 Hashimoto K, Lever WF. An electron microscopic study on pemphigus vulgaris of the mouth and the skin with special reference to the intercellular cement. *The Journal of investigative dermatology* 1967; **48**: 540-52.

19 Shu E, Yamamoto Y, Aoyama Y *et al.* Intraperitoneal injection of pemphigus vulgaris-IgG into mouse depletes epidermal keratinocytes of desmoglein 3 associated with generation of acantholysis. *Archives of dermatological research* 2007; **299**: 165-7.

20 Aoyama Y, Kitajima Y. Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3-depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells. *The Journal of investigative dermatology* 1999; **112**: 67-71.

21 Calkins CC, Setzer SV, Jennings JM *et al.* Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. *The Journal of biological chemistry* 2006; **281**: 7623-34.

22 Bannon LJ, Cabrera BL, Stack MS *et al.* Isoform-specific differences in the size of desmosomal cadherin/catenin complexes. *The Journal of investigative dermatology* 2001; **117**: 1302-6.

23 Aoyama Y, Owada MK, Kitajima Y. A pathogenic autoantibody, pemphigus vulgaris-IgG, induces phosphorylation of desmoglein 3, and its dissociation from plakoglobin in cultured keratinocytes. *European journal of immunology* 1999; **29**: 2233-40.

24 Kawasaki Y, Aoyama Y, Tsunoda K *et al.* Pathogenic monoclonal antibody against desmoglein 3 augments desmoglein 3 and p38 MAPK phosphorylation in human squamous carcinoma cell line. *Autoimmunity* 2006; **39**: 587-90.

25 Futamura S, Martins C, Rivitti EA *et al.* Ultrastructural studies of acantholysis induced in vivo by passive transfer of IgG from endemic pemphigus foliaceus (Fogo Selvagem). *The Journal of investigative dermatology* 1989; **93**: 480-5.

26 Takahashi Y, Patel HP, Labib RS *et al.* Experimentally induced pemphigus vulgaris in neonatal BALB/c mice: a time-course study of clinical, immunologic, ultrastructural, and cytochemical changes. *The Journal of investigative dermatology* 1985; **84**: 41-6.

27 Bystryn JC, Grando SA. A novel explanation for acantholysis in pemphigus vulgaris: the basal cell shrinkage hypothesis. *Journal of the American Academy of Dermatology* 2006; **54**: 513-6.

Chapter 3

Aggregation of IgG and desmosomal proteins in lesional mucosa of pemphigus patients

D.A.M. Oktarina, M.F. Jonkman and H.H. Pas

Center for Blistering Diseases, Department of Dermatology,
University Medical Center Groningen, University of Groningen,
Groningen, the Netherlands

Abstract

Background. Mucosal pemphigus vulgaris is a subtype of PV with autoantibodies against desmoglein 3 (Dsg3) only. Pemphigus foliaceus (PF) is characterized by autoantibodies to Dsg1. In a previous study we have analyzed the nature of the depositions in skin of patients with PV and of patients with PF.

Objectives. To investigate the IgG deposition patterns in mucous membranes and to correlate these with the distribution of desmosomal and non-desmosomal components.

Patients/Methods. We analyzed the distribution of IgG and desmosomal adhesion molecules in four mucosal epithelia from patients with PV oris and three biopsies of non lesional mucosa of PF patients by double staining immunofluorescence.

Results. In mucosal PV in basal cells beneath the blister we found clusters with the same composition—IgG, Dsg3 and PG-–as in healthy mucosal PV skin. Above the blister these clusters were also found but here in addition a second type of cluster was present that contained IgG and all desmosomal components. In non-blistering PF mucosa we found the same clusters—IgG, Dsg1 and PG—as in PF skin.

Conclusions. In PV and PF mucosa the same clusters are present as in the skin. In lesional PV mucosa additional clusters are present that we conclude to represent aggregated desmosomes. That aggregated Dsg1 is present in the lower layers of the mucosa questions current knowledge on cadherin distribution in oral mucosa.

Introduction

Mucosal dominant pemphigus vulgaris (PV) is an autoimmune blistering disease which is characterized by the loss of cell-cell adhesion, called acantholysis, in the mucous membranes. The clinical characteristics of this subtype of PV are flaccid blisters and erosions in mucous membranes non-keratinized stratified squamous epithelium, most times the oral cavity but also other mucosa of cornea, nasal cavity, laryngo-pharynx, esophagus, anal canal, vagina, and the internal portion of the lips may become affected, without any skin involvement. The type with pure oral involvement is named pemphigus vulgaris oris (PV oris). The opposite of mucosal PV is pemphigus foliaceus (PF). PF patients are characterized by blistering of the skin only and have no mucosal involvement. Mucosal PV is characterized by autoimmune IgG directed to desmoglein (Dsg) 3 but not to Dsg1 [1-3]. Histopathology of lesional epithelium shows a suprabasal blister level. In PV the first sign of pathogenesis is eosinophilic spongiosis later followed by acantholysis, the loss of cell-cell contact, and intraepidermal separation. PV oris patients have blisters on mucosal tissue but not in the skin. Mahoney *et al.* explained this by what they called the desmoglein compensation hypothesis which was already suggested by Burge *et al.* in 1993 [4,5]. The basic idea behind this hypothesis is that the level of blistering is determined by the desmoglein make-up of the desmosomes and the antigen-specificity of the immune response. If the IgG is directed to one single desmoglein all cells that express desmosomes with both Dsg1 and Dsg3 will be protected against acantholysis. Blistering will therefore occur at the site where the targeted Dsg is the only expressed Dsg in the desmosome. In PF, where the IgG is directed to Dsg1, this is the subcorneal layer where Dsg3 is absent. In mucosal PV this will be in the basal and suprabasal layer where Dsg1 is absent. If the IgG is directed to both Dsg1 and 3, as in mucocutaneous PV, then also the skin will blister between the basal and suprabasal layer.

In the previous chapter we have investigated the IgG deposition patterns in the skin of pemphigus patients. We showed that the IgG selectively dislocates the targeted desmoglein outside of the desmosomes into specific aggregates or clusters. Thus in the skin of mucosal PV skin the desmosomes become depleted of Dsg3, but this does not disturb the functioning of the skin as this remains sturdy and healthy in these patients. In PF skin desmosomes become depleted of Dsg1, what leads to widening between cells and acantholysis in the subcorneal layer. In this chapter we will look at the mucosal tissue of these same patients.

Materials and methods

Biopsies

Biopsies of four mucosal epithelia from patients with PV oris and three from non-lesional mucosa of PF patients were used for this study. The diagnosis was established in each patient based on clinical criteria and laboratory investigation, including histology, serum immunofluorescence, and direct immunofluorescence (DIF). All biopsies had been collected previously and stored at -80 °C. The ELISA anti-desmoglein profile was a further important

inclusion criterion as we included only PV patients with antibodies to Dsg3 and PF patients with antibodies to Dsg1. As control mucosa we used mucosa obtained from healthy donors.

Immunofluorescence microscopy

For DIF, cryostat sections of 4 μm thickness were cut and subsequently mounted on Polysine™ glass slides. Sections were encircled with a hydrophobic emulsion (PAP pen; Dako, Glostrup, Denmark) to keep antibody solutions in place during incubation and then air-dried before a fan for 15 minutes. For double staining of IgG deposits and specific adhesion proteins, the sections were washed in phosphate-buffered saline (PBS) (pH 7.3) and then incubated for 30 minutes in a moist chamber at room temperature with the primary (mouse monoclonal) antibody diluted in PBS containing 1% (w/v) ovalbumine (PBS/OVA).

The slides were washed with PBS for 15 minutes and then incubated with fluorescein-conjugated Fcγ-specific goat F(ab')2 anti-human IgG (Protos Immunoresearch, Burlingame,CA, U.S.A) in PBS/OVA in combination with Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes Eugene, OR, U.S.A) as secondary steps for 30 minutes. If the primary antibody was a rabbit polyclonal we used a FITC-conjugated donkey anti-rabbit IgG (Protos Immunoresearch, Burlingame, CA, USA) as second step. The slides were washed again with PBS for 15 minutes and then counterstained with 2 ug/ml bisbenzimide in PBS for five minutes. After a final 5 minutes PBS wash the sections were coverslipped using SlowFade® antifade reagent (Molecular Probes, Invitrogen, USA). For double staining with two different mouse monoclonals we used Zenon® Mouse IgG Labeling Kits Alexa Fluor®488 and Alexa Fluor®568 (Molecular Probes, Invitrogen, USA) by following technical protocols from the company. All slides were examined at 40 times magnification with a Leica DMRA fluorescence microscope. For selective excitation and emission, various filter cubes (Leica) were used, including the L5 (green), the A (blue) and the TX2 (red). Images were recorded using a Leica DFC 350FX digital camera (Leica Microsystems AG, Wetzlar, Germany). Gain and gamma were kept at 1.0 to keep the images as natural as possible. To obtain maximal information the levels of the separate channels were adjusted afterwards using Photoshop 7.0 software to mimic longer exposure times.

Antibodies

Desmoglein 1 was stained with Dsg1-P23 (dilution 1:20) or 27B2 (dilution 1: 40), desmoglein 2 with 10G11 (dilution 1:2), desmoglein 3 with Dsg3-G194 (dilution 1:40), desmocollin 1 with U100 (dilution 1:40) and desmocollin 3 with U114, diluted in 1:5 (all from Progen Immunodiagnostika, Heidelberg, Germany). Plakoglobin was stained with 15F11 (dilution 1:1000, Sigma-Aldrich, Missouri , USA), desmoplakin I/II with DP 1&2-2.15 (dilution 1:100, Roche Diagnostics, Mannheim, Germany) , β-catenin with 9G2 (dilution 1:50, Biotrend, Cologne, Germany), keratin 5 with BL18 (dilution 1:2000, gift of Dr. P. Ogden, Dundee, Schotland), and keratin 14 with RCK107 (dilution 1:10, (gift of Dr. F. Raemakers, Maastricht, the Netherlands). Plakophilin 3 staining was done with PKP3-270.6.2 (dilution

1:50, Progen Immunodiagnostika, Heidelberg, Germany) and E-cadherin with NCH-38 (dilution 1:100, Dako Cytomation, Denmark)

Results

Aggregation of IgG and desmosomal components in lesional PV oris mucosa

In lesional PV mucosa clustering of IgG was seen below and above the blister. As in pemphigus skin, Dsg3 and PG again had lost their normal smooth membrane distribution and had aggregated to dots that colocalized (Figure 1a to c). In this buccal mucosal epithelium the split had formed between the second and third cell layer, but the two cells deep layer beneath the split was keratin 14 positive defining these cells as undifferentiated proliferative keratinocytes (Figure 1d), confirming the split level as classical for PV. The big difference with non-lesional skin was that the basal cells seemed to have lost most of their desmosome components. The desmosome components Dsc3 and DP were not lost in the first suprabasal keratin-14-positive cells, although DP was more diffusely spread than normal extending from the cell membrane into the cytoplasm (Figure 1e to h), suggesting that the first suprabasal cells were turning acantholytic. Adherens junctions proteins E-cad and B-cat were normally present in the first two cell layers (Figure 1i, j).

Also the layers above the blister contained such Dsg3 dots, but now the aggregation that was seen included all desmosomal components. This can best be seen in a second lesional mucosa biopsy (Figure 2). The IgG here was deposited in a coarse dotted or granular pattern consisting of both elongated and more round shapes. Double staining showed colocalization of IgG with Dsg3 and PG (Figure 2a to f) but also with DP and Dsc3 (Figure 2g to l) and with other components like PKP3 (not shown).

Sections used for immunofluorescence were restained with hematoxylin and eosin, and the images were digitally overlaid with the fluorescent image. The elongated shapes colocalized with areas of intercellular widening, while more round aggregates overlayed fragments that seemed to separate from the cells (Figure 3). As in the first biopsy the basal cells had lost expression of desmosomal components but in contrast in this particular biopsy the basal cells were also almost devoid of the Dsg3/PG clusters.

Aggregation of IgG and desmosomal components in PF mucosa

Fine dots of IgG were visible in the lower layers of the mucosa. Double staining revealed that at these same positions Dsg1 and PG were present (Figure 4). No Dsg1 was seen outside the dots and the higher layers of the mucosa were devoid of Dsg1. Dsg3 did not colocalize (Figure 4). HE staining was inconclusive on widening. Further electron microscopic studies have to be awaited.

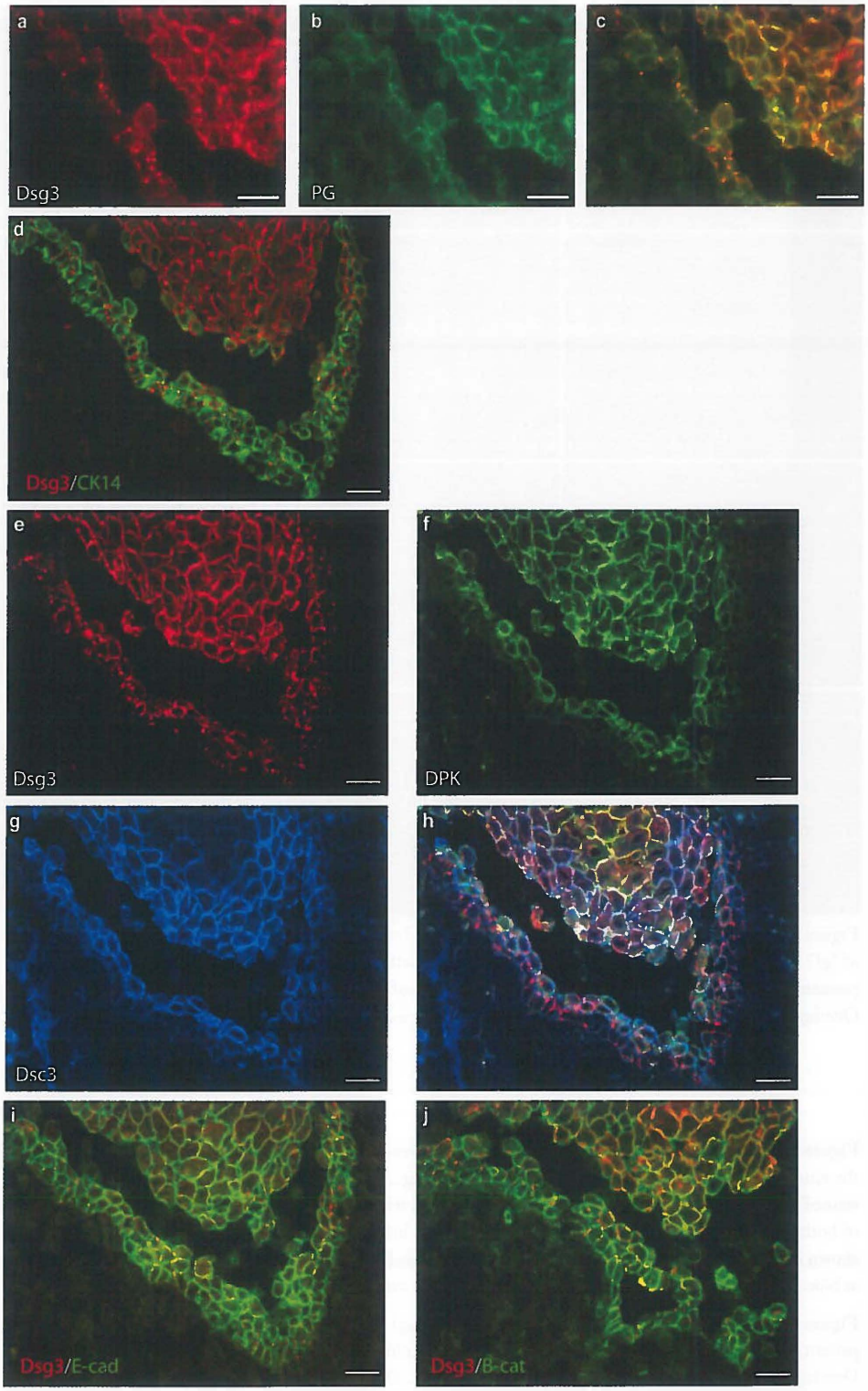
Discussion

Here we demonstrate that not only in the skin but also in the mucous membranes of patients with pemphigus vulgaris (PV) the *in vivo* bound IgG manifests in a granular pattern of dots, in contrast to the smooth *in vitro* pattern seen when serum of a patient is brought onto a section of skin. We show that two types of clusters are present, clusters with the same composition as in skin, and above the blister a second type of clusters that contain IgG, Dsg3, PG and other desmosomal components.

The second type of clusters is present in lesional or acantholytic tissue samples. Apart from IgG these aggregates contain all components that are normally found in the desmosomes of these layers. This suggests that in contrast to the first type of clusters these clusters consist of condensed complete desmosomes. Roughly two shapes of clusters were seen: elongated 'rice grain' patterns and more rounded clusters. Overlay images showed that the elongated shapes were present between the cells in areas that demonstrated widening of the interdesmosomal spaces between cells, a condition named spongiosis that normally is associated with extreme fluid filling of the space between the cells. In some of these clusters the immunofluorescence fine-pattern perfectly overlaid parallel aligned cell-cell bridging structures. The more rounded clusters may represent similar structures in the z-direction, but as they were observed more often in the layers just above the blister, they probably reflect remains of intercellular desmosomes that have been pinched off from the cell membrane under the pathologic traction. Our interpretation of this second type of clusters is that they likely arise as a secondary effect to spongiosis. As such they are not part of the pathological mechanism leading to acantholysis but rather represent collateral damage due to the blistering process.

In the previous study, the clusters in uninvolved skin in mucosal PV represent a situation where all desmosomal components including the majority of the PG, but with the exception of Dsg3, keep their normal distribution over the cell membrane. This indicates that the remaining desmosomes must be virtually devoid of Dsg3. This reminiscences the experiments of Aoyama *et al.* who witnessed depletion of Dsg3 from desmosomes in DJM-1 cells after incubation with pemphigus-IgG [6]. Thirty hours after incubation, the cell membrane Dsg3 staining had almost completely disappeared leaving Dsg3-depleted desmosomes. They proved that this result was due to the anti-Dsg3 antibodies in the serum by repeating their experiments with pure anti-Dsg3 monoclonal antibodies that gave the same effect [7]. The depletion of Dsg3 in DJM-1 cells, similar to the redistribution of Dsg3 in uninvolved PV-skin study, was not followed by acantholysis, which as argued by Caldelari could be due to the expression of compensating Dsg1 in the DJM-1 cells [8]. In mucosa where Dsg1 is supposed to

Figure 1. (Opposite page) Aggregates of IgG, Dsg3 and PG in lesional PV mucosa. The same aggregates as found in non-lesional skin were also present in lesional oral mucosa and were most prominent beneath the blister. (**a**) Dsg3, (**b**) PG and (**c**) overlay. The split had formed between the second and third layer above the keratin 14 positive compartment (**d**). Triple staining demonstrated that the basal layer, that still contained Dsg3/PG/IgG clusters, had lost most of its DP and Dsc3 expression, in contrast to the suprabasal layer were DP and Dsc3 were still present. (**e**) Dsg3, (**f**) DP, (**g**) Dsc3 and (**h**) overlay of **e**, **f** and **g**. Expression of adherens junction proteins E-cad (**i**) and B-cat (**j**) was normal in both layers. White bar is 20 micrometers.


a
Dsg3
b
PG
c
d
Dsg3/CK14
e
Dsg3
f
DPK
g
Dsc3
h
i
Dsg3/E-cad
j
Dsg3/B-cat

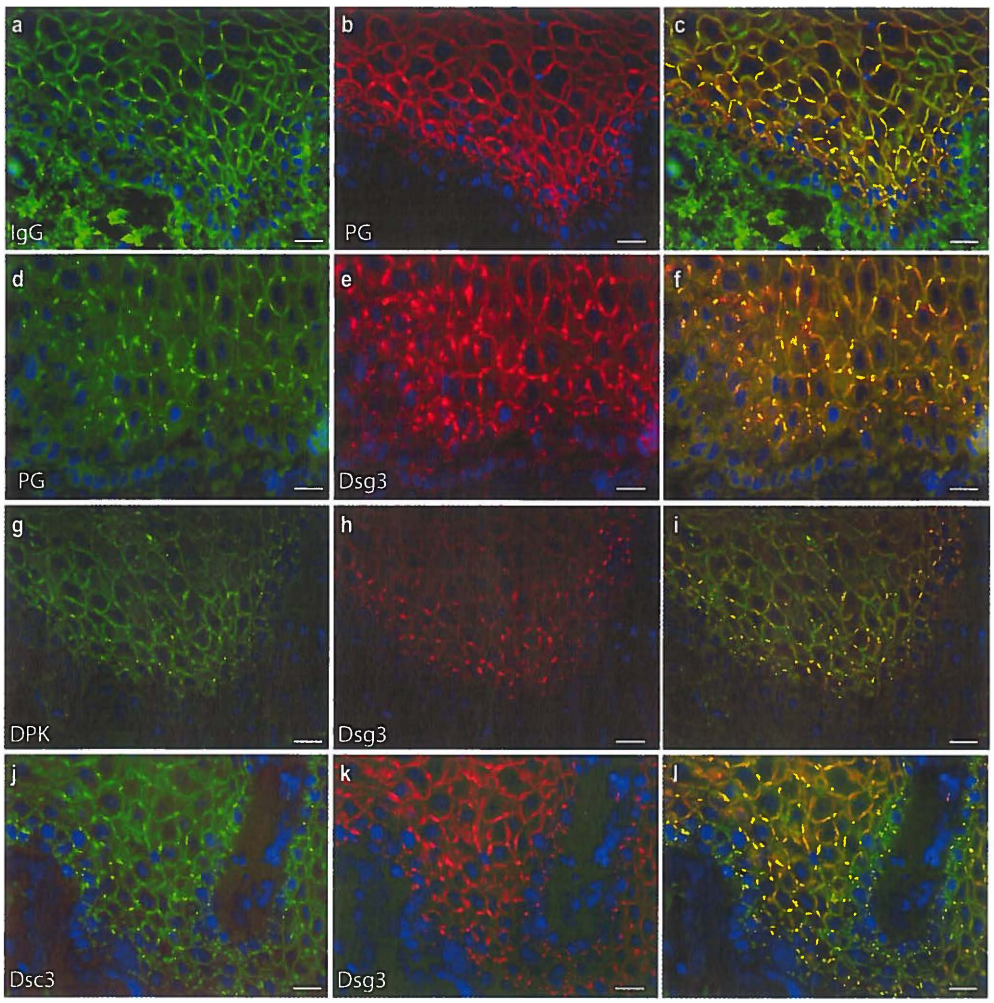


Figure 2. Clusters in lesional mucosa contain additional desmosomal components. Above the blister a second type of IgG aggregate was present that co-localized not only with Dsg3 and PG but also with other desmosomal components. (**a** to **c**) Overlay of IgG and PG. (**d** to **f**) Overlay of PG and Dsg3. (**g** to **i**) Overlay of DP and Dsg3. (**j** to **l**) Overlay of Dsc3 and Dsg3. Nuclei are in blue. White bar is 20 micrometers.

Figure 3. (Opposite page, top) Overlay of immunofluorescence and hematoxylin/eosin staining images localizes the elongated type of clusters in areas of cellular widening. A lesional mucosal tissue section where Dsg3 had been stained by immunofluorescence (**a**, detail **d**) was restained with hematoxylin/eosin (**b**, detail **e**). The recorded images of both stainings were digitally overlaid (**c**, detail **f**). The black rectangle in **a**, **b** and **c** denotes the detailed area as shown in **d**, **e** and **f**. The IF recorded image (**a**, **d**) was digitally processed such that the Dsg3 clusters became visible as blue dots that could easily be distinguished in the final overlay image. White bar is 20 micrometers.

Figure 4. (Opposite page, bottom) Aggregates of IgG, Dsg1 and PG, but not Dsg3, in non-lesional mucosa of a PF patient. IgG aggregates (**a**, **d**, **g**) were present that co-localized with Dsg1 (**b**) and PG (**e**), but not with Dsg3 (**h**). Overlay of IgG and Dsg1 (**c**). Overlay of IgG and PG (**f**). Overlay of IgG and Dsg3 (**i**). Nuclei are in blue.

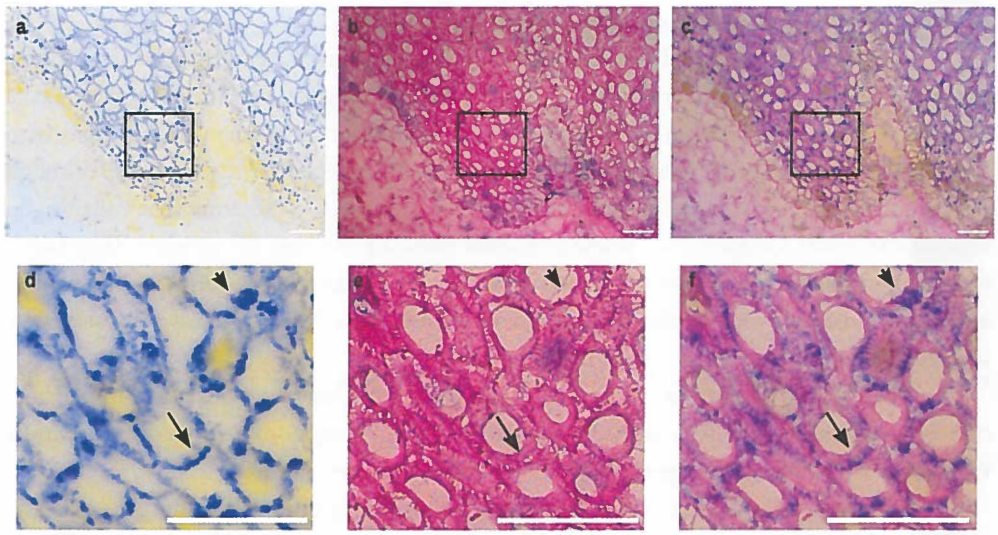


Figure 3. (Caption, opposite page)

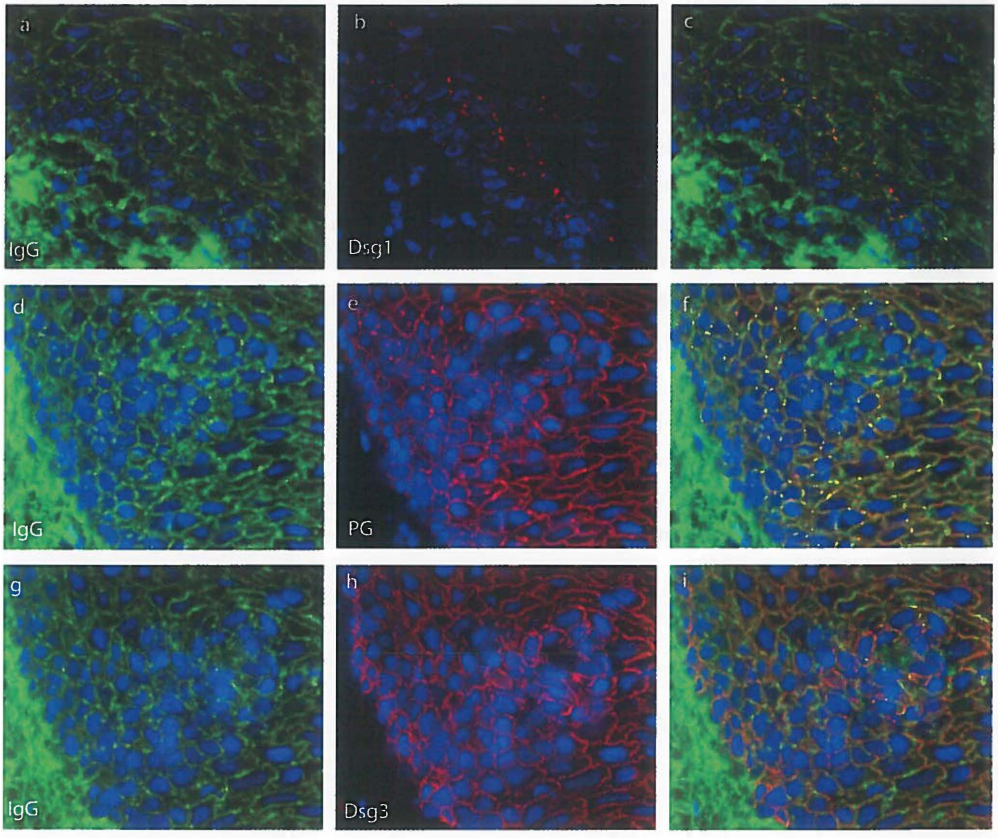


Figure 4. (Caption, opposite page)

be absent Dsg3 depletion will end in acantholysis.

Unexpectedly we found Dsg1 clustering in the lower layers of the mucosa. In the previous **chapter 2** we have described that intercellular widening between desmosomes occurs in the lower layers of the skin in PF patients and found that this widening correlated with Dsg1 clustering. Therefore intercellular widening can be expected in the mucosa. Although our biopsies were inconclusive there is supportive evidence in the literature that widening in mucosa can occur. Guedes *et al.* studied mucosal tissue of endemic PF patients. They found widening in the lower cell layers of oral mucosa, internal surface of the foreskin, uterine cervix, and vaginal wall while at the same time these tissues were clinically uninvolved similar to our patient. Dsg1 distribution was not addressed by them.

The finding of Dsg1 in the lower layers of the oral mucosa contradicts the current concept on Dsg1 distribution in the oral mucosa. Dsg1 is thought to be present in declining expression from the higher layers and to be absent in the lower layers.

If our observations are correct they raise a puzzling question. The compensation hypothesis of Stanley and Amagai explains blistering in mucosa through the absence of Dsg1. If Dsg1 is present in the lower layers why does it not compensate for Dsg3 loss in mucosal PV with anti-Dsg3 antibodies? If compensation would occur than we would not expect a suprabasal blister but instead a blister above the layers that express Dsg1. Furthermore also the row of tombstones would not be expected as the loss of the lateral desmosomes of the basal cells would be absent. Surprisingly a higher split without a row of tombstones is what we observed in two of our three PV oris biopsies. The third biopsy was from a patient with additional anti-BP230 antibodies, and we do not know if these latter antibodies are able to interfere with pemphigus pathogenesis. The two other biopsies did demonstrate a blister above the suprabasal layer (see Figure 1 for blistering level) in line with Dsg1 compensation. Currently the literature lacks data on the quantitative contribution of the diverse cadherin isoforms to the different desmosomes. Our data indicate that there is need for reinvestigating the expression patterns of the Dsg isoforms in the different types of mucosa as current concepts may be incorrect.

Acknowledgments

This work was in part supported by the Groningen Bernoulli Fund and a Schlumberger Foundation Faculty for the Future grant to DAMO.

References

1 Amagai M, Klaus-Kovtun V., Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67**: 869-77.

2 Stanley JR, Koulu L, Klaus-Kovtun V *et al.* A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 1986; **136**: 1227-30.

3 Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *J Clin Invest* 1984; **74**: 313-20.

4 Burge SM, Wilson CL, Dean D *et al.* An immunohistological study of desmosomal components in pemphigus. *Br J Dermatol* 1993; **128**: 363-70.

5 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J Clin Invest* 1999; **103**: 461-8.

6 Aoyama Y, Kitajima Y. Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3-depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells. *J Invest Dermatol* 1999; **112**: 67-71.

7 Yamamoto Y, Aoyama Y, Shu E *et al.* Anti-desmoglein 3 (Dsg3) monoclonal antibodies deplete desmosomes of Dsg3 and differ in their Dsg3-depleting activities related to pathogenicity. *J Biol Chem* 2007.

8 Caldelari R, de Bruin A, Baumann D *et al.* A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris. *J Cell Biol* 2001; **153**: 823-34.

Chapter 4

Desmoglein 1 ectodomain is present in basement membrane zone deposits of IgG and complement in pemphigus erythematosus

D.A.M. Oktarina, D. Kramer, G.F.H. Diercks, M.F. Jonkman and H.H. Pas

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

Submitted

Abstract

Background. Pemphigus foliaceus (PF) is characterized by circulating autoantibodies to desmoglein 1 (Dsg1) that deposit intraepidermal in a partly granular pattern. Sometimes additional granular depositions of IgG and complement are seen along the epidermal basal membrane zone (BMZ). This combined pattern has in the past been connected with pemphigus erythematosus (PE). Recently antibodies to BP230 and a 190 kDa antigen were described in patients with combined intraepidermal and BMZ depositions.

Objective. To analyze (i) the composition of the BMZ deposits, (ii) the immune response for additional autoimmune targets and, (iii) if the combined deposition pattern corresponds clinically with PE.

Patients/Methods. We investigated biopsies and sera of patients with (n=3) or without (n=14) BMZ IgG deposition. We retrospectively analyzed patient files for clinical manifestations.

Results. The BMZ deposits differed from the intraepidermal deposits. Plakoglobin was absent but Dsg1 was present although not as the full-length molecule but rather as a shed part of the ectodomain. The Dsg1 containing deposits were located around the lamina densa. All three patients reacted weakly by ELISA to BP230, however no BP230 was found in the BMZ deposits. The typical PE butterfly facial rash was definitely found in two patients. ANA antibodies were absent. Remarkably all three patients were initially misdiagnosed as having psoriasis and had received light therapy.

Conclusions. The presence of Dsg1 ectodomain fragments along the BMZ explains why the IgG deposits here. The shed Dsg1 ectodomain indicates the presence of a to PF additional pathogenic mechanism. Clinically BMZ Dsg1 ectodomain deposition corresponds with PE.

Introduction

Pemphigus foliaceus (PF) is an autoimmune skin disease characterized by subcorneal blistering and intraepidermal deposition of IgG antibodies directed against desmoglein 1 (Dsg1). Occasionally additional deposition of IgG is present along the epidermal basement membrane zone (BMZ), and this combined pattern has in the past been correlated with the pemphigus variant pemphigus erythematosus (PE), originally known as Senear-Usher syndrome. Based on its clinical manifestations PE was initially suggested by Senear and Usher to be a condition where pemphigus vulgaris (PV) is combined with lupus erythematosus (LE) [1]. For PE these are eruptions on the face in the typical butterfly pattern or severe seborrheic dermatitis. Typical pemphigus Nikolsky-positive blisters are found that are usually small and flaccid, but also thick greasy scales and yellowish crusts can be seen [2]. When insights into the differences between PV and PF crystallized PE was not classified with PV anymore but instead considered an early or not generalized form of PF [3]. With immunofluorescence becoming a diagnostic parameter the association with LE revived. Reports appeared in which a so-called 'lupus-band' deposition was discovered in sun-exposed skin areas of PE patients together with anti-nuclear antibodies (ANA) as in LE [4]. Later papers however showed less clinicopathological concurrency with LE [5,6], as ANA antibodies appeared often absent [7,8], and the overall significance of this became disputed as it appeared that ANA antibodies were also present in a high percentage of the normal population [9]. Although it is clear that in occasional cases LE can present simultaneously with pemphigus the gross of the PE patients do not by far meet the criteria for SLE as published by the American College of Rheumatology [10,11]. Therefore what is called PE today should be separated from the sparse cases of actual concurrent LE and PV/PF, and the basic teaching books nowadays consider PE just a localized form of PF [12].

The diagnostic criteria for PE do remain somewhat obscure. Clinically PE is suspected in non-generalized disease with either symmetric face distribution or seborrhoic appearance [13-16]. In both PE and PF indistinguishably the typical subcorneal blister is found and in both circulating autoantibodies against Dsg1 are present. In contrast to PF however biopsies of PE patients often demonstrate the 'lupus-band' phenomenon in which a coarse granular deposition of IgG and complement is found along the BMZ in addition to the typical intercellular space (ICS) deposition [4,5]. As this is present in a high percentage of patient biopsies (up to 60%) [5], it must represent a unique immunopathological aspect of PE. As in LE the mechanism of this BMZ deposition in PE remains unclear.

In 2003, Karlhofer *et al.* reported the presence of IgG antibodies to the bullous pemphigoid antigen BP230 additional to Dsg1 in PF patients with combined ICS and BMZ deposition, what suggested that the BP230 antigen could immunopathologically be involved in the BMZ deposition [17]. This would imply that a hemidesmosomal antigen would possible be cause of the BMZ deposits. They also found reactivity to a 190-kDa antigen that by immunoblot had the same apparent molecular weight as periplakin.

We have recently investigated the ICS deposition in PF and have shown that the

IgG deposits in a granular pattern around the cells, especially in the lower layers (Oktarina et al, submitted). This granular deposition is caused by IgG induced aggregation of Dsg1 and plakoglobin (PG). Here we have extended this study to patients with combined ICS and BMZ deposition, and we demonstrate that ectodomain fragments of Dsg1 are present in the BMZ deposits described before as a 'lupus band'. The finding of Dsg1 in this 'lupus-band' appeared unique in these patients as we did not find Dsg1 in the lupus-band of LE patients.

Materials and methods

Patient sera and biopsies

Among the PF patients of whom we had stored biopsies in our -80°C tissue bank three had biopsies with additional granular deposition of IgG along the BMZ. A further 14 that did not have BMZ deposition served as PF controls. All patients had been seen at our own department. The diagnosis in all 17 patients had been established on clinical criteria and laboratory investigation, including histology, and immunofluorescence of skin (DIF) and serum on monkey oesophagus (IIF). ELISA demonstrated for all patients IgG antibodies to Dsg1 but not to Dsg3. The skin specimens had been immediately frozen in liquid nitrogen and stored in our -80°C freezer after diagnostic purposes. The serum samples had been taken at the time of biopsy and had also been stored at -80°C. We further included four biopsies of LE patients, one with systemic LE and three with chronic discoid LE. Skin obtained from breast reduction of healthy individuals was used as control skin.

Immunofluorescence microscopy

The procedure for immunofluorescence staining and image collection has been described before in detail [18]. For visualization of adhesion molecules we used the following monoclonals; Dsg1-P23 and Dsg1-P124 (Dsg1 ectodomain), 27B2 and DG3.10 (Dsg1 endodomain), AK23 (desmoglein 3 ectodomain), U100 (desmocollin (Dsc) 1), U114 (Dsc3), 15F11 (PG), 279 (BP230), AE11 (periplakin) and LH7.2 (type VII collagen). As DG3.10 also stains the endodomain of Dsg2 we checked all biopsies with Dsg2-specific monoclonal 10G11 but no detectable expression of Dsg2 was found, thus DG3.10 staining in this study represents only the ectodomain of Dsg1. Double staining of IgG and adhesion molecules IgG was performed with fluoresceinthiocyanate (FITC)-conjugated Fcγ-specific goat F(ab')2 anti-human IgG (Protos Immunoresearch, Burlingame, CA, U.S.A) and Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes Eugene, OR, U.S.A) as secondary steps. For complement C3c we used FITC-conjugated rabbit anti-human C3c (DAKO, ITK Diagnostics, Glostrup, Denmark) For double staining with two different mouse monoclonals we used Zenon® Mouse IgG Labelling Kits Alexa Fluor®488 and Alexa Fluor®568 (Molecular Probes, Invitrogen, USA) by following technical protocols from the company. Nuclei were counterstained with 2 μg/ml bisbenzimide.

Enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis

ELISA's for detection of anti-Dsg1 and anti-BP230 autoantibodies were performed using commercially available Dsg1 and BP230 ELISA Kits (MBL, Nagoya, Japan) according to the manufacturer's protocols. The cut-off values for considering samples positive were 9 U/ml for the BP230 ELISA and 20 U/ml for the Dsg1 ELISA. The procedure for immunoblotting has been extensively described before [19]. As substrate an epidermal extract was used that was prepared as described before [20].

Results

Granular deposition of IgG and C3c along the BMZ

All 17 patients had biopsies that displayed intraepidermal deposition of IgG in a partly granular character in line with PF. In the three patients with additional IgG BMZ deposition that IgG had a coarse granular appearance and colocalized with the BMZ C3c deposition. Of the 14 other patients 11 also had C3c deposition along the BMZ, which in all cases was granular although the pattern ranged between more coarse and fine dust-like. However none of these had IgG BMZ deposition. Of the three patients that had BMZ IgG we had in total nine biopsies stored in our tissue bank. In six of these IgG was present along the BMZ. The three other biopsies only had intraepidermal IgG deposition (Table 1). In contrast, from the 14 other patients we had in total 51 biopsies stored and none of these had BMZ IgG deposition.

Patient	Skin biopsy	Biopsy site	IgG BMZ deposition	IgG ICS deposition
1	healthy	upper leg	+	+
1	lesional	upper leg	-	+
2	healthy	upper arm	+	+
2	perilesional	lower leg	+	+
2	perilesional	inner arm	+	+
2	perilesional	inner arm	+	+
2	perilesional	upper leg	-	+
3	perilesional	leg	+	+
3	lesional	flank	-	+

Table 1. Patient biopsy IgG deposition characteristics. Positive and negative findings are denoted by respectively + and -.

Dsg1 is present in the BMZ deposits of IgG and C3c

As Dsg1 is the autoantigen in PF we stained our biopsies with anti-Dsg1 monoclonal Dsg1-P23. The staining overlapped the IgG and C3c depositions demonstrating that Dsg1 was not only present in the intraepidermal deposits but also in the BMZ deposits (Figure 1 a-c and b-f). To confirm our observation we stained with three other anti-Dsg1 monoclonal antibod-

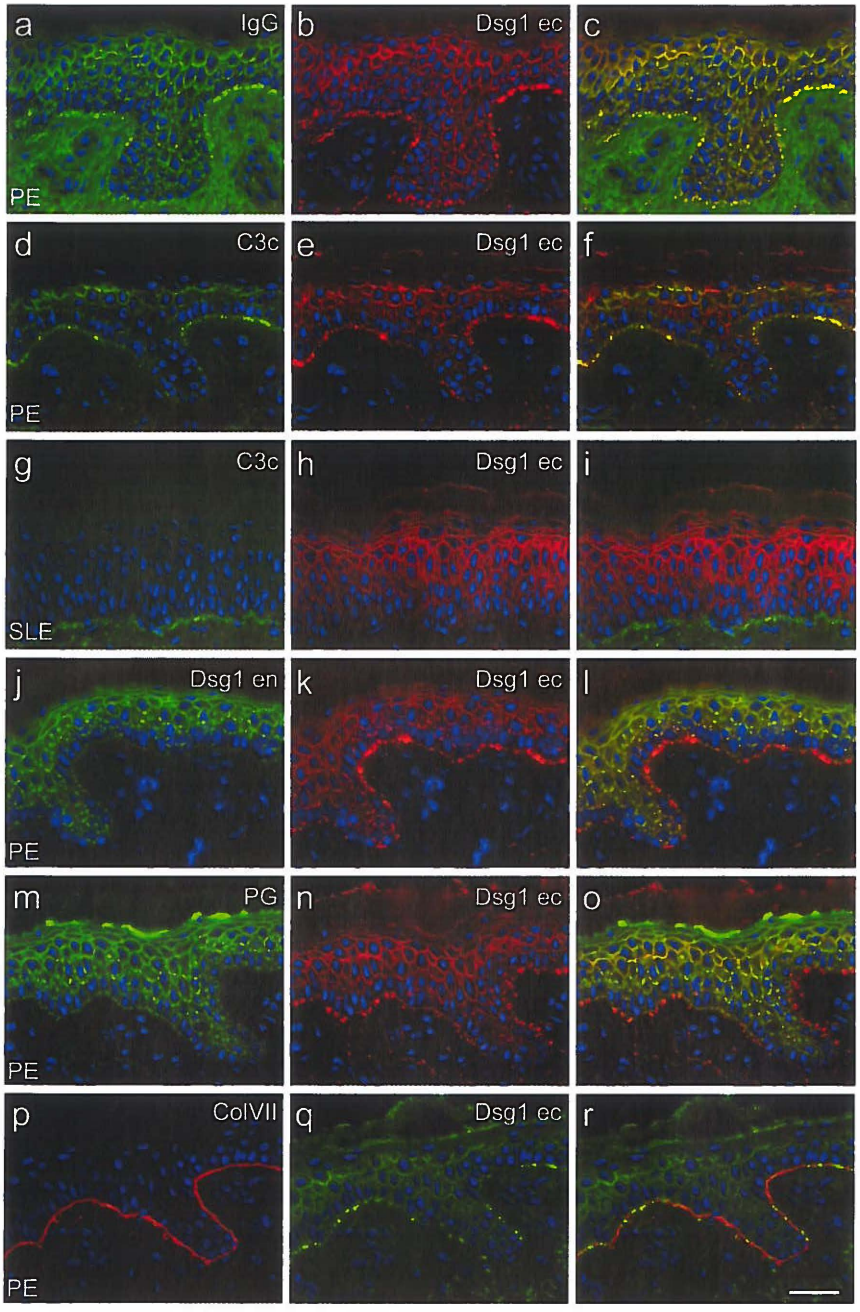


Figure 1. Dsg1 ectodomain epitopes are present in the granular BMZ deposition of IgG and complement. Double staining immunofluorescence of PE skin reveals colocalization along the BMZ of IgG (a) and C3c (d) with the ectodomain of Dsg1 (b, e). In contrast no Dsg1 ectodomain (h) is found in the BMZ complement deposition (g) in LE skin. The BMZ Dsg1 ectodomain deposits (k,n) did not contain the Dsg1 endomain (j) or plakoglobin (m). The granular Dsg1 deposition (q) was scattered above, under and at the level of type VII collagen (p). The right column are overlays of the reds and greens at the left. Nuclei are in blue. All images have the same magnification. The white bar depicts 40 micrometers.

ies, the ectodomain specific Dsg1-P124, and the endodomain specific 27B2 and DG3.10. Dsg1-P124 colocalized with the BMZ IgG but 27B2 and DG3.10 (Figure 1j-l) did not. Other desmosmal cadherins, Dsg3, Dsc1 and 3 were all absent in the BMZ depositions (not shown). We recently described that the granular intraepidermal deposits in PF consist of IgG, Dsg1 and PG and therefore we stained our biopsies also for PG. PG appeared to be only present in the epidermal deposits but not along the BMZ (Figure 1 m-o). The absence of the cytoplasmic PG and the endodomain of Dsg1 in the deposits made it likely that the deposits were located outside the cells and possibly not connected with the cell membrane of the basal keratinocytes. A detailed view of the BMZ shows that the BMZ clusters are located underneath the intraepidermal clusters (Figure 2). We therefore mapped the position of the deposits by double staining with monoclonals to the adhesion molecules type XVII collagen, laminin-332 and type VII collagen that map to different levels of the BMZ. Most deposits colocalized with type VII collagen with some laying beneath and more occasionally above indicating that they are scattered over and around the lamina densa (Figure 1 p-r). In the lupus band of the biopsies of the four LE patients no Dsg1 was found thus the presence of Dsg1 in such band is exclusive to PF (Figure 1 g-i).

Serum antibodies to BP230 and a 190 kDa molecule

To rule out any relationship with LE all sera were assayed for ANA antibodies, but none of them reacted positive. As previously antibodies to BP230 and a 190 kDa protein were reported in patients with combined ICS and BMZ deposition we next assayed the sera of our patients for such antibodies. The sera of the three patients with additional BMZ deposition reacted positive in a BP230 ELISA, albeit it with low index values, but the other 14 sera reacted negative (Table 2). On Immunoblot one out of 17 sera displayed binding to a band at approximately 190 kDa and this was one with a combined staining pattern (Table 2). Immunoblot however could not confirm the presence of anti-BP230 as not even weak binding was at the position of BP230. Also salt-split skin analysis was negative. The identity of the 190 kDa molecule is unclear but has been suggested to be periplakin that stains at approximately the same position in Immunoblot. To investigate if BP230 and periplakin were possibly involved in the BMZ deposition we double stained biopsies for IgG and respectively BP230 and periplakin but did not find any evidence for this (not shown).

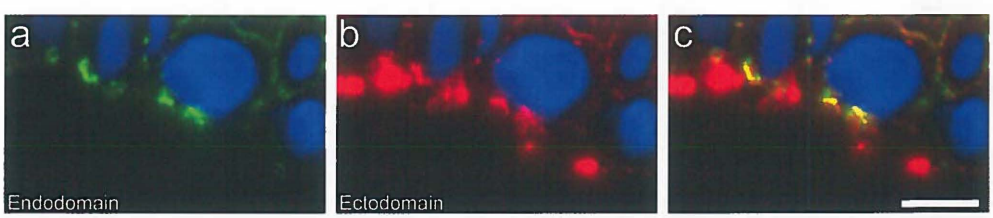


Figure 2. Dsg1 containing deposits along the BMZ. Deposits that contain only the Dsg1 ectodomain fragment but not the Dsg1 endomain are located underneath deposits that contain the full-length Dsg1. The latter deposits are typical PF aggregates of IgG, PG and Dsg1 that already form low in the basal cells as described before (Oktarina et al, submitted). Nuclei are in blue. The white bar depicts 10 micrometers.

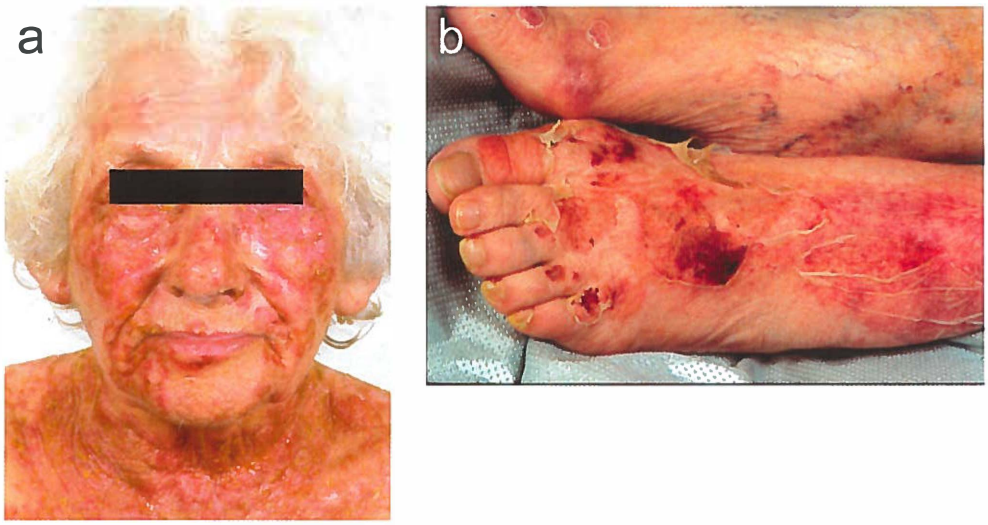


Figure 3. (**a**) The typical PE facial butterfly eruption of patient #1, and (**b**) extensive lesions six weeks after ending UVB therapy of patient #3.

Patient	Clinical appearance	ELISA Dsg1 index	Granular BMZ IgG	Granular BMZ C3c	BMZ Dsg1	ELISA BP230 index	Immunoblot 190 kDa
1	PE	257	+	+	+	18	-
2	PE	237	+	+	+	25	+
3	PE	198	+	+	+	17	-
4	PF	131	-	-	-	2	-
5	PF	170	-	+	-	0	-
6	PF	169	-	+	-	2	-
7	PF	146	-	-	-	1	-
8	PF	183	-	+	-	3	-
9	PF	218	-	+	-	0	-
10	PF	109	-	+	-	2	-
11	PF	239	-	+	-	0	-
12	PF	243	-	+	-	0	-
13	PF	193	-	+	-	4	-
14	PF	187	-	+	-	1	-
15	PF	91	-	-	-	0	-
16	PF	261	-	+	-	4	-
17	PF	160	-	+	-	0	-

Table 2. Summarized clinical and laboratory patient data. Positive and negative findings are denoted by respectively + and -.

Features of PE in the patients with combined ICS BMZ deposition.

We re-evaluated the patient files to see if the combined deposition patterns matched PE rather than PF. For two patients (# 1 and 2) we could retrieve that they showed the typical malar butterfly eruption (Figure 3a). For the third patient unfortunately insufficiently specified file information and photo documentation on facial eruption was available. A remarkable finding from the patient files was that of all 17 patients these three patients had been misdiagnosed elsewhere for psoriasis and had received light therapy before being referred to us. Patients # 1 and 2 had been treated with PUVA what for both patients led to severe exacerbation. Patient #3 received UVB therapy for four months what resulted in extensive lesions (Figure 3b) .

Discussion

The composition of the BMZ deposits and accompanying serum characteristics make clear that PF patients who have depositions of IgG both intraepidermal and along the BMZ should be considered as a separate entity rather than as having an early, more localized, manifestation of PF. The latter is unlikely as the 14 control PF patients in a total of 51 biopsies did neither show any deposition of IgG along the BMZ or abnormal serum reactivity, while six of the nine biopsies of the other three patients were positive for BMZ IgG and their sera displayed anti-BP230 reactivity in ELISA. As the clinical manifestations of at least two of the patients matched the historical criteria set for PE it is clear that in PE there is unique pathology next to acantholysis. Any overlap with LE was absent, as the patients did not have ANA antibodies or otherwise fulfilled LE criteria.

The finding of Dsg1 epitopes in the BMZ depositions was a surprise but also most logical, Dsg1 being the primary antigen in PF. We found that both the Dsg1-P23 and the Dsg1-P124 ectodomain directed monoclonals bound the BMZ deposits. There is no doubt that both recognize Dsg1 as both stain normal human skin in the correct distribution pattern, both stain PF skin with in the rearranged distribution pattern, and furthermore both recognize Dsg1 by immunoblot. As endodomain epitopes were absent and the deposits were located to far from the basal cell membrane for the ectodomain of Dsg1 to bridge, this leaves three alternatives for the recognized molecule along the BMZ. (i) It is a shed part of the ectodomain of Dsg1 or (ii) it is the alternative spliced soluble form of Dsg1 whose sequence partly overlaps the sequence that was used to generate both monoclonals [21] or (iii) it is a molecule that immunologically cross reacts with Dsg1. We feel that the latter is unlikely seen that both different monoclonals bind the BMZ deposits, while on normal human skin both produce the typical Dsg1 staining pattern. As for the second option, the alternative spliced form is consistently present in skin and contains the pathogenic EC1 epitope recognized by the majority of PF patient sera [21,22]. Therefore if the alternative spliced form would be origin to the BMZ deposition such would be expected in the majority of the PF patients, what is

not found. That the deposits are initiated by the shed ectodomain or part of that would imply that in PE a proteolytic mechanism is present that is not found in PF.

In the past it has been argued that BMZ IgG deposition in PE could be related to sun exposure in analogy with LE. Chorzelski described BMZ deposition in the sun-exposed facial skin of supposed PE patients [4]. Giannetti in a later study demonstrated that this finding was specific to sun-exposed areas. Of five patients he biopsied lesional skin from both the face and the back. Where in four of five facial biopsies BMZ IgG deposition was present in contrast all five biopsies of the back were BMZ-negative [7]. Our BMZ-positive biopsies were taken from arm and leg, what for these three patients were UV-exposed sites as all three had received extensive whole body light therapy. We therefore think it highly possible that UV-radiation is connected with the development of the BMZ deposition. This would imply that UV in some way induces a pathological process in which Dsg1 molecules are proteolytically attacked and loose their ectodomains. When these diffuse out into the dermal compartment they will form immune complexes with the circulating anti-Dsg1 antibodies and deposit. As this does not happen in 'ordinary' PF, PE patients likely somehow are predisposed to develop such pathomechanism. That such pathomechanism also would underlie the severe exacerbations observed in our PUVA-treated patients is less clear. Case reports have demonstrated UVB induced lesions in PE but an older study on seven patients showed that also in PF acantholysis can be provoked by UV irradiation [23-25].

As in a previous study we found a reactivity to BP230 although not by immunoblot but by ELISA. It has been suggested that these anti-BP230 antibodies could be cause of the BMZ deposition, for instance the granular pattern could result from anti-BP230 IgG binding to the cytoplasmic part of BP230 in damaged keratinocytes [17]. Our results indicate that this is unlikely, as immunomapping revealed that the granular deposits are located around the lamina densa rather than at the level of hemidesmosomes. Second, when we stained our biopsies for BP230 we could not find any evidence that BP230 was present in the depositions. Although it is striking that from our 17 patients only the three with combined deposition had a weakly positive ELISA reaction we at this moment have no clue about the significance or meaning of these antibodies. Also the band of 190 kDa we found in one of the patients and that was reported before in three patients remains an enigma, and as for BP230 we found no evidence for the presence of periplakin in the depositions.

Considering all the results presented in this study, we conclude that PE is a subform of PF, immunologically characterized by unique Dsg1 ectodomain containing granular EBMZ immune deposits. In PE a to PF additional pathogenic mechanism is active that might be provoked by UV radiation. Importantly, PE is a separate disease not to be confused with a combination of LE and pemphigus.

Acknowledgments

This work was in part supported by the Groningen Bernoulli Fund and a Schlumberger Foundation Faculty for the Future grant to DAMO.

References

1 Senear FE, Usher B. An unusual type of pemphigus combining features of lupus erythematosus. *Arch Dermatol Syphilol* 1926; 13: 761-81.

2 Steffen C, Thomas D. The men behind the eponym: Francis E. Senear, Barney Usher, and the Senear-Usher syndrome. *Am J Dermatopathol* 2003; 25: 432-6.

3 Perry HO, Brunsting LA. Pemphigus Foliaceus. Further Observations. *Arch Dermatol* 1965; 91: 10-23.

4 Chorzelski T, Jablonska S, Blaszczyk M. Immunopathological investigations in the Senear-Usher syndrome (coexistence of pemphigus and lupus erythematosus). *Br J Dermatol* 1968; 80: 211-7.

5 Amerian ML, Ahmed AR. Pemphigus erythematosus. Senear-Usher syndrome. *Int J Dermatol* 1985; 24: 16-25.

6 Jablonska S, Chorzelski T, Blaszczyk M *et al.* Pathogenesis of pemphigus erythematosus. *Arch Dermatol Res* 1977; 258: 135-40.

7 Giannetti A. Immunofluorescence studies in the Senear-Usher syndrome. *Arch Dermatol Forsch* 1974; 248: 287-96.

8 Bean SF, Lynch FW. Senear-Usher syndrome (pemphigus erythematosus). Immunofluorescent studies in a patient. *Arch Dermatol* 1970; 101: 642-5.

9 Tan EM. Special antibodies for the study of systemic lupus erythematosus: an analysis. *Arthritis Rheum* 1982; 25: 753-6.

10 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.

11 Malik M, Ahmed AR. Concurrence of systemic lupus erythematosus and pemphigus: coincidence or correlation? *Dermatology* 2007; 214: 231-9.

12 Amagai M. Pemphigus. In: *Dermatology-2nd Edition* (Bolognia JL, Jorizzo JL, Rapini PR, eds), 2 edn.: Elsevier, 2007.

13 Jablonska S, Chorzelski T, Maciejowska E. The scope and limitations of the immunofluorescence method in the diagnosis of lupus erythematosus. *Br J Dermatol* 1970; 83: 242-7.

14 Lyde CB, Cox SE, Cruz PD, Jr. Pemphigus erythematosus in a five-year-old child. *J Am Acad Dermatol* 1994; 31: 906-9.

15 Maize JC, Green D, Provost TT. Pemphigus foliaceus: a case with serologic features of Senear-Usher syndrome and other autoimmune abnormalities. *J Am Acad Dermatol* 1982; 7: 736-41.

16 Willemsen MJ, De Coninck AL, De Raeve LE *et al.* Penicillamine-induced pemphigus erythematosus. *Int J Dermatol* 1990; 29: 193-7.

17 Karlhofer FM, Hashimoto T, Slupetzky K *et al.* 230-kDa and 190-kDa proteins in addition to desmoglein 1 as immunological targets in a subset of pemphigus foliaceus with a combined cell-surface and basement membrane zone immune staining pattern. *Exp Dermatol* 2003; 12: 646-54.

18 Vodegel RM, Jonkman MF, Pas HH *et al.* U-serrated immunodeposition pattern differentiates type VII collagen targeting bullous diseases from other subepidermal bullous autoimmune diseases. *Br J Dermatol* 2004; 151: 112-8.

19 Pas HH, Kloosterhuis GJ, Heeres K *et al.* Bullous pemphigoid and linear IgA dermatosis sera recognize a similar 120-kDa keratinocyte collagenous glycoprotein with antigenic cross-reactivity to BP180. *J Invest Dermatol* 1997; 108: 423-9.

20 Pas HH. Immunoblot assay in differential diagnosis of autoimmune blistering skin diseases. *Clin Dermatol* 2001; 19: 622-30.

21 Mouquet H, Farci S, Joly P *et al.* A truncated alternative spliced isoform of human desmoglein 1 contains a specific T cell epitope binding to the pemphigus foliaceus-associated HLA class II DRbeta1*0102 molecule. *J Immunol* 2006; 177: 6517-26.

22 Yokouchi M, Saleh MA, Kuroda K *et al.* Pathogenic epitopes of autoantibodies in pemphigus reside in the amino-terminal adhesive region of desmogleins which are unmasked by proteolytic processing of prosequence. *J Invest Dermatol* 2009; 129: 2156-66.

23 Jacobs SE. Pemphigus Erythematosus and Ultraviolet Light. A Case Report. *Arch Dermatol* 1965; 91: 139-41.

24 Cram DL, Winkelmann RK. Ultraviolet-induced acantholysis in pemphigus. *Arch Dermatol* 1965; 92: 7-13.

25 Igawa K, Matsunaga T, Nishioka K. Involvement of UV-irradiation in pemphigus foliaceus. *J Eur Acad Dermatol Venereol* 2004; 18: 216-7.

Chapter 5

Possible role of endocytosis in pemphigus foliaceus skin

D.A.M. Oktarina, D. Kramer, M.F. Jonkman and H.H. Pas

Centre for Blistering Diseases, Department of Dermatology, University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands

Abstract

Background. Pemphigus foliaceus (PF) is one of the two main forms of pemphigus and is characterized by circulating IgG to the desmosomal cadherin desmoglein (Dsg) 1 and subcorneal blistering of the skin. Previously we have shown that PF IgG induces aggregation of Dsg1, plakoglobin (PG) and IgG outside of desmosomes, what in immunofluorescence of PF patient skin visualizes as a granular IgG deposition pattern with a limited number of coarse IgG aggregates per cell. Older immunoelectron microscopic studies have suggested that in PF skin endocytosis of desmosomes takes place.

Objectives. To verify this endocytosis and, if present, to investigate its relation with the IgG aggregates.

Patients/Methods. We performed double immunofluorescence staining on 10 skin biopsies of six PF patients for the following molecules: IgG, Dsg1 and 3, desmocollin 1 and 3, PG, desmoplakin, plakophilin 3, early endosomal marker 1 (EEA1) and cathepsin-D.

Results. EEA1 did not colocalize with the IgG aggregates in the basal and suprabasal layers, but did contact them in the layers above. Endosomes were present in these cells of the upper epidermis, and contained IgG, Dsg1 and PG but not other cadherins or plaque components. Endocytosis was only present in lesional skin, but not in non-lesional skin. Our results confirm that endocytosis of IgG bound components takes place in skin of PF patients.

Conclusions. Based on the molecular composition of the endosomal cargo we conclude that these endosomes do not take up complete desmosomes but instead clear the IgG/Dsg1/PG aggregates from the cell membrane.

Introduction

Pemphigus is an autoimmune blistering disease of the skin and mucous membranes, which is characterized by a loss of cell-cell adhesion, known as acantholysis. Immunopathologically, pemphigus is characterized by the circulating IgG which binds in the keratinocytes cell surface in an intercellular substance (ICS) pattern. There are two main classical forms of pemphigus: pemphigus vulgaris (PV) and pemphigus foliaceus (PF). PV evokes flaccid blisters in the mucosa, and also in the skin in more than half of the cases. Histologically it is characterized by suprabasal splitting with acantholytic basal cells. PF evokes fragile blisters, scales, and erosions only in the skin due to the superficial splitting in the epidermis. Pathogenic autoantibodies react specifically against desmoglein 1 (Dsg1) in the PF sera, while autoantibodies directed to Dsg3 and sometimes also to Dsg1 are recognized in PV sera [1-4].

Dsg 1 and Dsg3 are calcium-dependent transmembrane glycoproteins (cadherins) of desmosomes, which homophilic bind neighbouring keratinocytes. Desmosomes can be found in the tissues that need mechanical resistance, such as skin and heart. Desmosomes consist of transmembrane cadherins, including Dsg 1-4 and desmocollins (Dsc) 1-3, and intracellular plaque proteins, including plakoglobin (PG), desmoplakin (DP), and plakophillins 1-4 (PKP). The latter proteins link the cytoplasmic tails of the desmosomal cadherins to the keratin intermediate filaments [5,6].

The pathomechanism of blister formation in pemphigus is still a matter of debate. There are many theories and speculations to elucidate the pathology of blistering in pemphigus [7-13]. Several studies support the theory that the binding to free Dsg3 molecules leads to their internalization into endosomes. Sato *et al.* in 2000 observed internalization of the non desmosomal Dsg3 as a primary effect of IgG binding [14]. In 2006 Calkins *et al.* demonstrated that the internalized IgG-Dsg3 complex colocalized with endosome and lysosome markers [15]. Using a biotinylation process, they observed that the soluble Dsg3 in the cell surface was first depleted, followed by the insoluble Dsg3 [15]. Later Delva *et al.* in 2008 found the pathway of the internalization of the Dsg3 [16]. They showed that the IgG-Dsg3 complex is internalized through a clathrin- and dynamin-independent pathway and that this event is connected with pathogenic IgG [16].

The studies above all addressed IgG (anti-Dsg3) induced Dsg3 internalization, however only one study investigated the fate of Dsg1 after PF IgG (anti-Dsg1) binding. Cirillo *et al.* in 2007 showed the internalization of Dsg1 and that this reduced keratinocyte adhesion. They demonstrated, similar to PV IgG acting on Dsg3, that PF reduced the amount of soluble pool of Dsg1, but in contrast did not result in the early depletion of Dsg1 from the adhesion complex [17].

The above studies on endocytosis of PV and PF IgG and desmogleins all have been done on monolayer cell culture systems. What is known about patient skin? Already in 1964 Wilgram *et al.* analysed PF patient skin (n=7) by electron microscopy and noted that in all specimens 'peculiar intracytoplasmic bodies' of 200 nm diameter were present that were most frequently seen adjacent to acantholytic areas [18]. In 1989 Iwatsuki *et al.* studied the

fate of IgG in epidermal sheets obtained from mucocutaneous PV patients [19]. These sheets were obtained by use of the Nikolsky phenomenon near a blister, and contained three to eight cell layers, thus probably the lowest layer represented the suprabasal layer. When the IgG was labelled with ferritin particles and the sheets were incubated at 37°C the ferritin was internalized and electron microscopy showed cytoplasmic vesicles containing ferritin and desmosomal structures. In 1999 they studied patient skin and claimed the presence of vesicles containing detached desmosomes [20]. Tada and Hashimoto in 1996 studied both PV and PF skin by immunoelectron microscopy and found what they called curvicircular bodies in PF but not in PV skin [21]. These were found in the higher, but not in the lower layers of the epidermis. These structures labelled positive for Dsg1, PG, IgG and connexin 43, and did not contain attachment plaques or inserting tonofilaments, and were hypothesized to be IgG-bound desmosome-gap-junction complexes that transformed into curvicircular structures.

We reported in a previous study clustering of Dsg1, PG and IgG in the skin of PF patients. In this study we have investigated if endocytosis is present in skin of PF patients and, if so, if this is related with these clusters.

Material and methods

Patient biopsies

Ten biopsies of 6 PF patients were included in this study. The diagnosis in all 6 patients had been established on clinical criteria and laboratory investigation, including histology, and immunofluorescence of skin (DIF) and serum on monkey esophagus (IIF). The skin specimens had been immediately frozen in liquid nitrogen and stored in our -80°C freezer after diagnostic purposes. Skin obtained from breast reduction was used as healthy control skin.

Immunofluorescence microscopy

The procedure for immunofluorescence staining and image collection has been described before in detail [22]. For visualization of adhesion molecules we used the following monoclonals; Dsg1-P23 and Dsg1-P124 (Dsg1 ectodomain), 27B2, DG3.10, 18D4 and B-11 (Dsg1 endodomain), Dsg3-G194 (Dsg3), U100 (Dsc1), U114 (Dsc3), 15F11 (PG), DP2.15 (DP), and PKP3-270.6.2 (PKP3).Early endosomal antigen was stained with EEA1, while cathepsin D was stained with CTD-19. Double staining of IgG and adhesion molecules IgG was performed with fluoresceinthiocyanate (FITC)-conjugated Fcγ-specific goat F(ab')2 anti-human IgG (Protos Immunoresearch, Burlingame, CA, U.S.A) and Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes Eugene, OR, U.S.A) as secondary steps. For complement C3c we used FITC-conjugated rabbit anti-human C3c (DAKO, ITK Diagnostics, Glostrup, Denmark). For double staining with two different mouse monoclonals we used Zenon® Mouse IgG Labeling Kits Alexa Fluor®488 and Alexa Fluor®568 (Molecular Probes, Invitrogen, USA) by following technical protocols.

Results

Endocytosis of IgG, Dsg1 and PG in the upper layer of lesional PF skin

Previously we have demonstrated that PF skin contains clustered IgG deposits. The clusters contain IgG, PG and Dsg1 and in areas of heavy clustering some DP and PKP3. PG was present in a clustered pattern through all epidermal layers (Figure 1A). When we stained lesional PF skin with EEA1 fine cytoplasmic dots were visible that represented endosomes and these were most prominent in the upper layers (Figure 1B). The overlay image of PG and EEA1 showed co-localization of the two antibodies in the superficial layers of the epidermis but not in the lower layers (Figure 1C).

In the spinous layer groups of endosomes moored on to the PG clusters, often symmetrically from two adjacent cells (Figure 2). Also endosomes containing PG were seen that left the clusters and moved into the cytoplasm (Figure 3A-C). In cells higher in the epidermis, underneath the blister, PG containing endosomes localized near the nucleus (Figure 3D-F). In these cells most of the clusters seemed to have disappeared. As the clusters contain Dsg1 and IgG next to PG we expected that both Dsg1 and IgG are endocytosed together with PG.

Double staining of EEA1 with the ectodomain of Dsg1 and of EEA1 with IgG showed colocalization of both IgG and Dsg1 with the cytoplasmic endosomes (Figure 4). Next we stained for the endodomain of Dsg1 and this was also present in these endosomes. Figure 5 shows colocalization of PG and the Dsg1 endodomain. When we stained non-lesional skin no colocalisation of endosomes with IgG, PG or Dsg1 was visible (not shown).

No endocytosis of other desmosomal cadherins or plaque components desmoplakin (DP) and plakophilin 3 (PKP3)

In older publications it was suggested that complete desmosomes are endocytosed. We also stained for other desmosomal components. Other cadherins Dsc1, Dsc3, and Dsg3 were absent in the Dsg1 containing endosomes (Figures 6 to 8).

As we previously demonstrated that in areas of heavy clustering also some PKP3 and DP co-concentrated we also double stained for Dsg1 and PKP3 and for Dsg1 and DP. Dsg1 and PKP3 double stained in the clusters however the endosomes that moved inwards from the clusters only contained Dsg1 (Figure 9). Thus PKP3 is not co-internalized with the IgG/PG/Dsg1 complex. Similarly DP also is not present in endosomes (Figure 10). Apparently they are disconnected from the Dsg1/PG during endocytosis.

Possibility of IgG breakdown in lysosomes

In order to understand the fate of the IgG, Dsg1, and PG complex after endosomal uptake, we double stained the lesional biopsies with IgG and cathepsin D as a lysosomal marker. Cathepsin D was present as fine cytoplasmic dots that were mainly present in the upper layers. However most of the IgG did not colocalize with the cathepsin dots, although occasionally

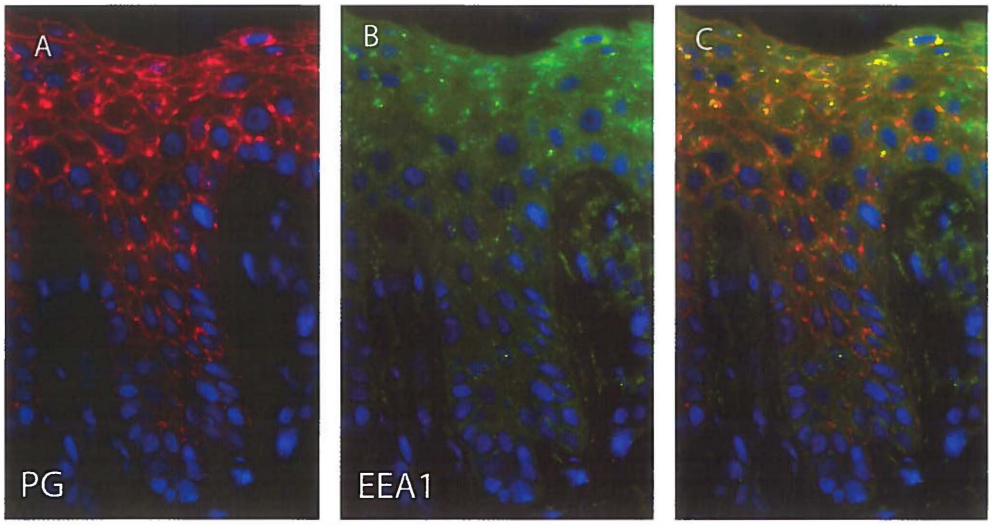


Figure 1. Endosomal activity in the upper layer of PF skin. Double staining of PG and EEA1 revealed that PG showed a cluster pattern throughout the epidermal layers **(A)**, while EEA1 staining was prominent in the upper layer, next to and just beneath the PF blister **(B)**. The difference of the two distributions can be clearly seen in the overlay image **(C)**.

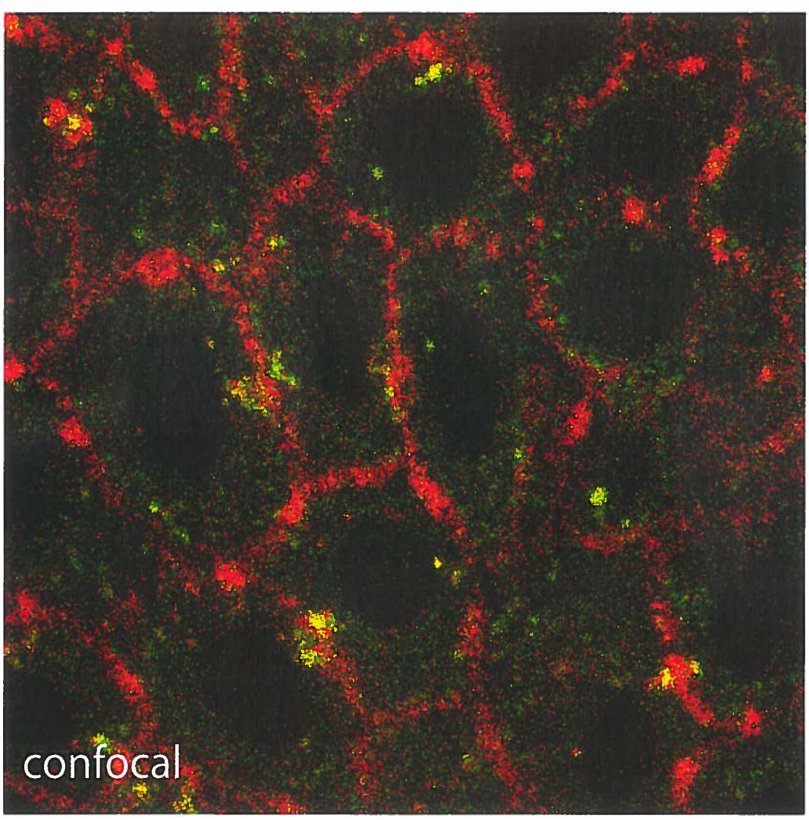


Figure 2. The confocal image showed that EEA1 (green) are moored on both sides of PG (red) clusters.

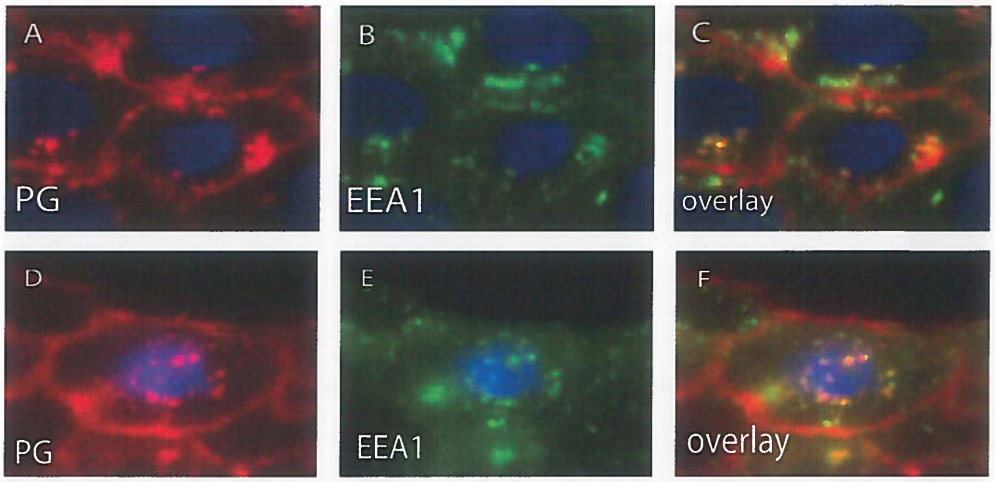


Figure 3. On both sides of PG clusters, EEA1 seemed to leave the membrane and move towards the nucleus. In the detailed pictures, PG was seen in a clustered pattern around the membrane **(A)** and EEA1 was present on both sides of the PG cluster **(B)**. The colocalization can be seen in the overlay image **(C)**. The EEA1 and PG intracellular clusters seemed to leave the membrane and move towards the nucleus **(D-F)**.

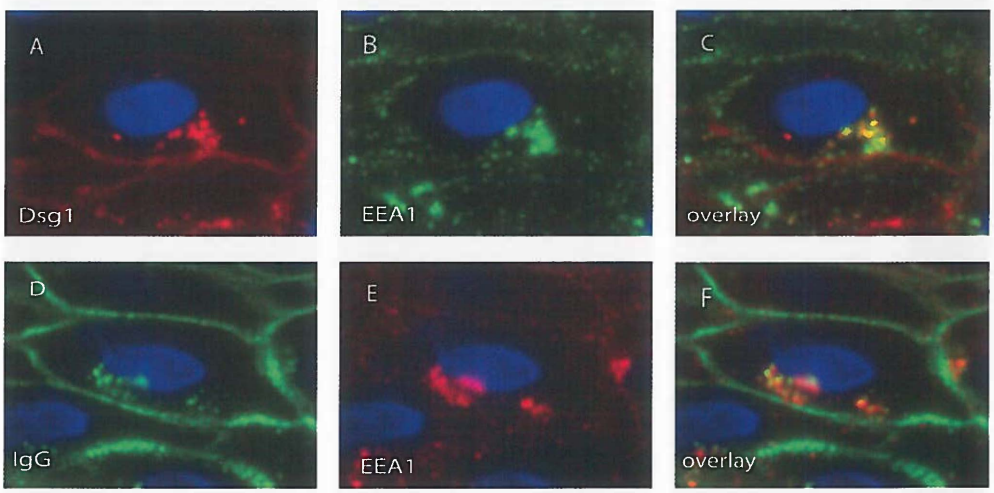


Figure 4. Ectodomain of Dsg1 and IgG were present in the endosomes. The ectodomain of Dsg1 was present in the intracellular clusters **(A)**, co-localizing with EEA1positive endosomes **(B)** and this can clearly be seen on the overlay image **(C)**. Also IgG co-localized **(D)** with the EEA1-labelled endosomes **(E)**. The colocalization of IgG and EEA1 can be clearly seen in the overlay image **(F)**.

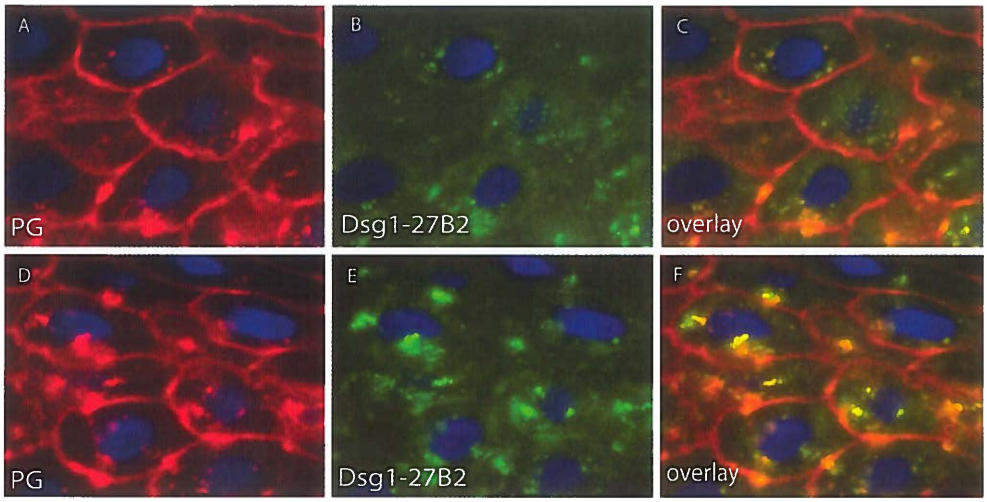


Figure 5. (Caption, opposite page)

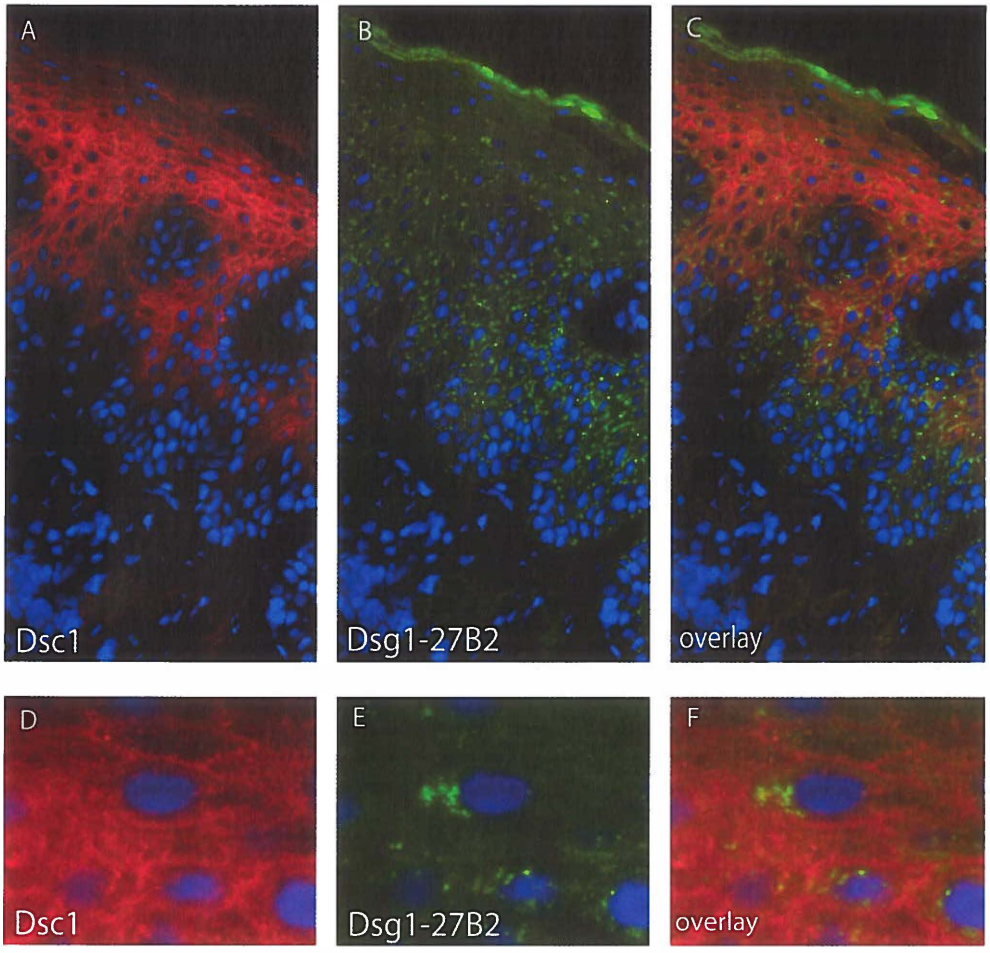
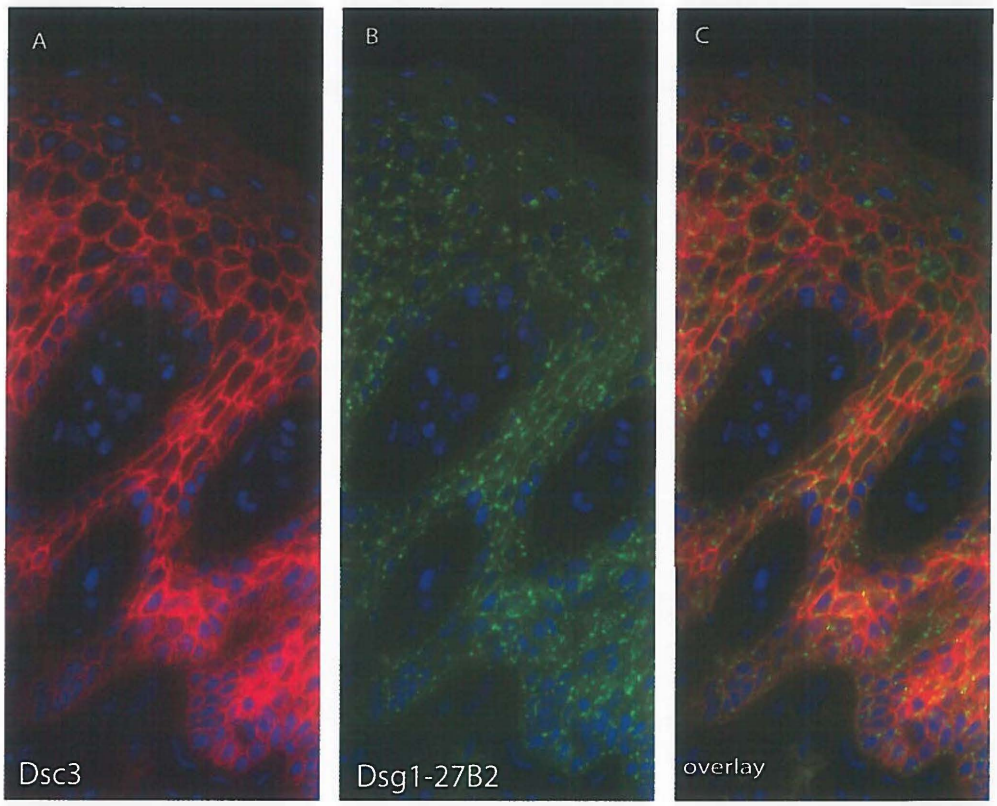


Figure 6. (Caption, opposite page)

Figure 5. (Opposite page, top) The endodomain of Dsg1 was also present in the endosomes. Double staining of PG (**A** and **D**) and the endodomain of Dsg1 (**B** and **E**) showed the colocalization (**C** and **F**) of the two components in the cell, exactly in the place where EEA1 was expressed.

Figure 6. (Opposite page, bottom) Dsc1 did not colocalize with Dsg1 clusters in endosomes. The double staining of the Dsc1 and the endodomain of Dsg1 showed that Dsc1 kept its smooth distribution pattern in all layers of the epidermis **(A)**, while the endodomain of Dsg1 was stained in an intracellular cluster pattern **(B)**. This can be seen clearly in the overlay image **(C)**, and also in the detailed pictures **(D-F)**.



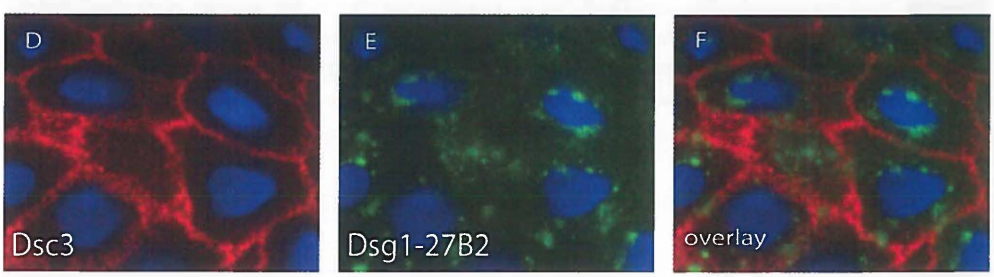


Figure 7. Dsc3 did not colocalize with Dsg1 clusters in endosomes. The double staining of the Dsc3 and the endodomain of Dsg1 showed that Dsc3 kept its smooth distribution pattern, mainly in the lower layers of the epidermis **(A)**, while the endodomain of Dsg1 was stained in an intracellular cluster pattern **(B)**. This can be seen clearly in the overlay image **(C)**, and also in the detailed pictures **(D-F)**.

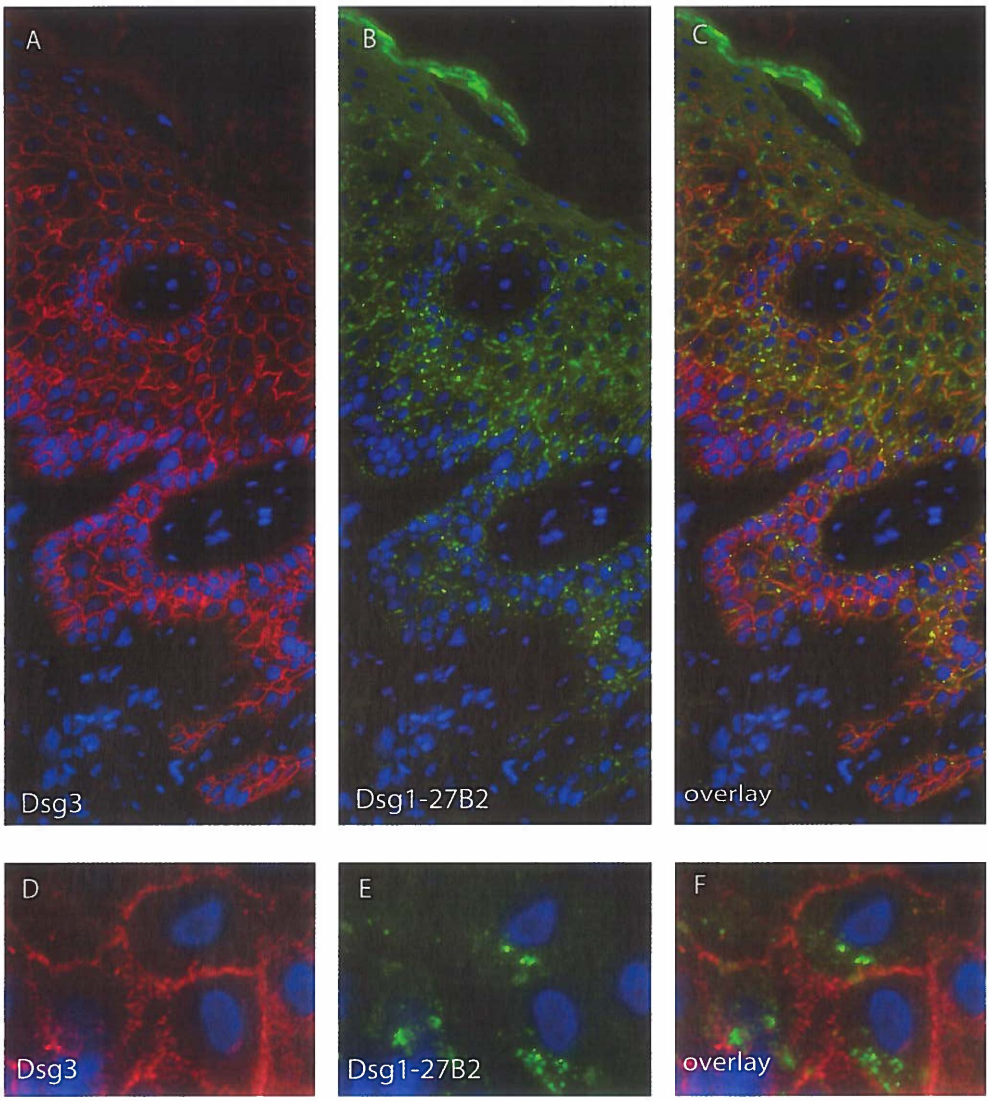


Figure 8. Dsg3 did not colocalize with Dsg1 clusters in endosomes. The double staining of the Dsg3 and the endodomain of Dsg1 showed that Dsg3 kept its smooth distribution pattern in the lower layers of the epidermis **(A)**, while the endodomain of Dsg1 was stained in an intracellular cluster pattern **(B)**. This can be seen clearly in the overlay image**(c)**, and also in the detailed pictures **(D-F)**.

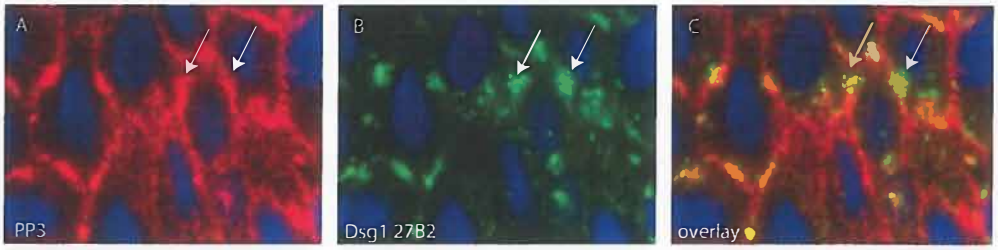


Figure 9. PKP3 did not colocalize with Dsg1 clusters in endosomes. The double staining of the PKP3 **(A)** and the endodomain of Dsg1 **(C)** showed that PKP3 did not co-internalized with the IgG/PG/Dsg1 complex. This can be seen in the overlay image **(B)**.

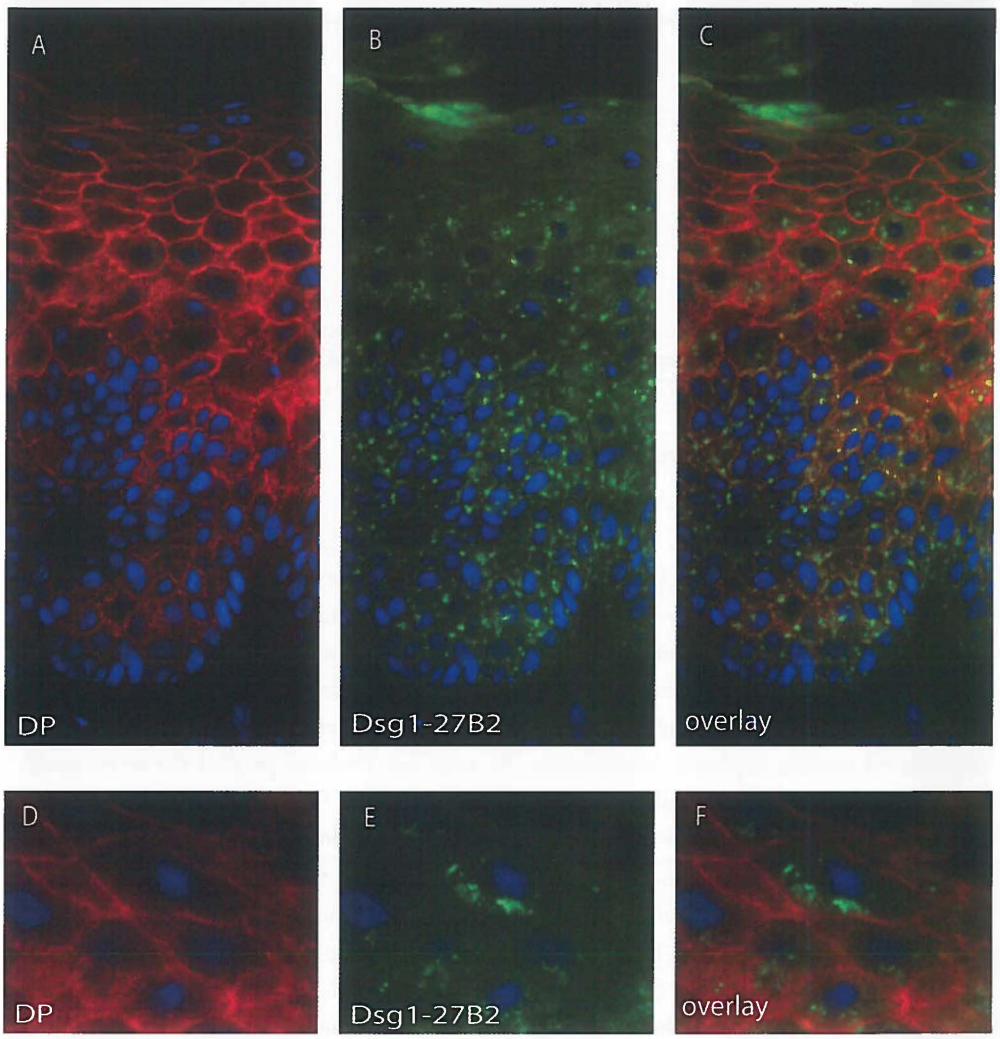


Figure 10. DP did not colocalize with Dsg1 clusters in endosomes. The double staining of the DP and the endodomain of Dsg1 showed that DP kept its smooth distribution pattern throughout the epidermis **(A)**, while the endodomain of Dsg1 was stained in an intracellular cluster pattern **(B)**. This can be seen clearly in the overlay image **(c)**, and also in the detailed pictures **(D-F)**.

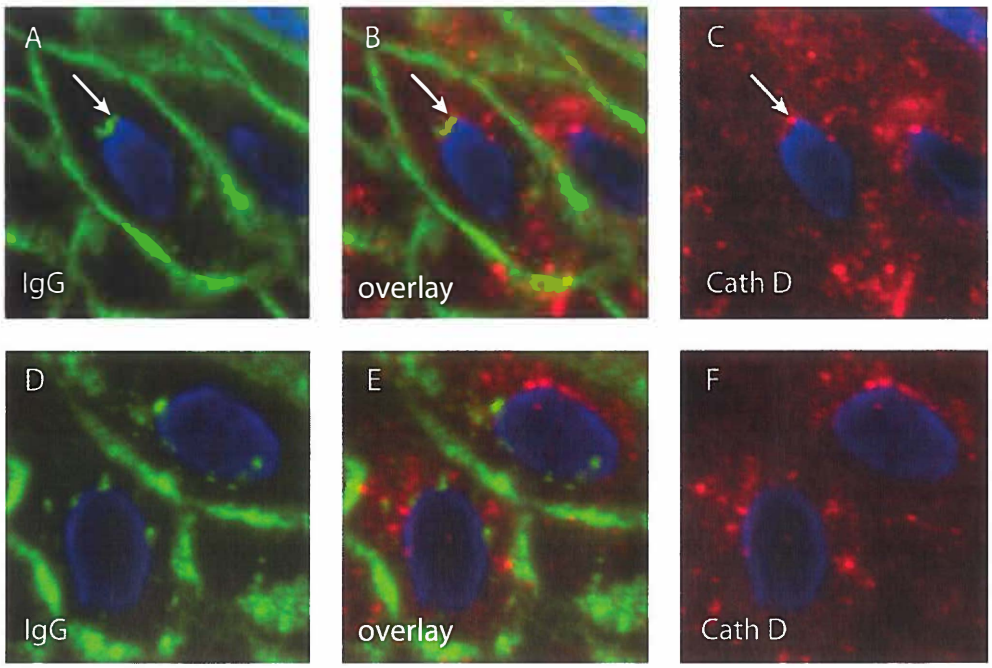


Figure 11. Occasionally IgG colocalized with cathepsin D positive lysosomes in the upper layers of the epidermis. The double staining of IgG and cathepsin D showed that the IgG clusters **(A** and **D)** were occasionally colocalized with the cathepsin D staining **(C** and **F).** This can be seen clearly in the overlay image **(B** and **C).**

colocalization was seen (Figure 11).

Discussion

Here we demonstrate that in lesional skin of PF patients the IgG induced clusters of IgG, PG and Dsg1 are removed by endocytosis. Older electron and immunoelectron studies had already suggested that endosomal uptake is active in PF skin and in these studies it was suggested that desmosomal structures were endocytosed. Our studies instead show that in the endosomes only those molecules are found that we in an earlier study demonstrated to be sequestered outside depleted desmosomes. As such our findings parallel the immunoelectron findings of Tada and Hashimoto who also found Dsg1, PG and IgG in their so-called curvicircular structures [21]. The connexion 43 they found could not be confirmed by us (not shown). As them we also found that endocytosis is absent in the lower epidermal layers but starts in the higher layers. Another similarity is that they did not find endocytosis in PV skin. Also Wilgram noted that the intracytoplasmic bodies that he saw seemed to be specific for PF as he did not see them in five PV skin specimens [23,24]. We studied our oral PV skin that contains decreased levels of clustered Dsg3 by double staining with EEA1 but did not find any signs of endocytosis as in PF skin (unpublished). We conclude that the endocytosis in PF skin does not represent internalization of desmosomes but that it is a process that clears the aggregates from the cell membrane. How do our findings relate to the electron microscopic

images from older studies? Iwatsuki described the contents of the cytoplasmic vesicles as detached desmosomes. Their pictures however are not that convincing and could also represent some aggregated material [19,20]. Tada and Hashimoto did not see attachment plaques or inserting tonofilaments [21]. We do not know the ultrastructure of our clusters. We before have shown that the clusters result from Dsg1 cross-linking by the IgG. Being a transmembrane protein the Dsg's are likely to remain floating in the membrane. As PG remains attached to the crosslinked Dsg1 the aggregates may well have some structure that could be interpreted as detached desmosomes. We therefore are fairly confident that our immunofluorescence observed endosomes represent the same cytoplasmic vesicles as in the older studies of Tada and Hashimoto in 1996 [21]. Future electron and immunoelectron studies should bring evidence of this.

How do our findings relate to anti-Dsg3 induced endocytosis of Dsg3 in cultured cells? There is abundant evidence that adding anti-Dsg3 IgG to cultured keratinocytes results in endocytosis of a IgG/Dsg3/PG complex [14-16,25,26]. As such these data on PV seem quite similar to ours as the desmosomal cargo does not contain other desmosomal components as desmoplakin [15]. Delva *et al.* investigated the endocytic pathway of Dsg3 internalization and concluded that it was not clathrin- or caveolae-mediated but rather a lipid-raft dependent pathway. So far we have not been able to find evidence for lipid-raft dependency of the endocytosis in our PF skin (preliminary results, not shown). In **chapter 1** we have demonstrated that clustering in PF starts already in the lower layer. However it is striking that no endocytosis is seen here. Endocytosis starts in the spinous layer and is visible in all cells above that layer. What phenomenon underlies this difference in endocytotic activity is unclear, but could be related with the differentiation status of the keratinocytes. Remarkably endocytosis is present close to the blister but is not seen in non-lesional skin. Endocytosis therefore may be related with blistering, and further research is definitely warranted. Berkowitz *et al.* demonstrated that p38 MAPK inhibitors inhibit endocytosis and that these same inhibitors also stop blistering in PV mouse models [25-28]. If indeed endocytosis is crucial to blistering then this raises the question if desmosomal depletion, leading to loss of desmosomes in Dsg3-deficient subcorneal cells underlies acantholysis, or that the uptake of IgG clustered material is also involved. At this moment we cannot answer this question. For future discussions it is also important to consider the time path of the endocytic process. Endocytosis is already observed several cell layers underneath the blistering level. It will take days before these cells reach the blistering level. The mere process of endocytosis therefore does not immediate result in acantholysis, which argues against a direct role for endocytosis as through cell signalling. Also further research into the nature of the clusters is needed. We observed that endocytosis often takes place from the same cluster symmetrically in two adjacent cells, and this might indicate that the cells are still connected through these clusters. Therefore these clusters might still have adhesive properties. In that case clearance of the clusters by internalization might indeed lead to loss of adhesion and acantholysis.

In the cell culture experiments the internalized IgG/Dsg3 complex is targeted for

degradation as they also colocalize with lysosomes. We addressed the possibility that in skin the lysosomes are also the destiny of the IgG/Dsg1/PG complex. This would seem most logical, as there is no function for IgG inside skin keratinocytes. Double staining of IgG with cathepsin D showed only occasionally colocalizing dots. We however do not know the rate of IgG digestion by lysosomes but we assume this is a fairly fast process. Therefore the IgG that has been taken up by lysosomes might rapidly disappear and not be traceable anymore by immunofluorescence. The occasional colocalization we observe might therefore be the moment that endosomes contact the lysosomes.

We conclude that our observations confirm older studies that witnessed endocytosis in skin of PF patients. However our results demonstrate that not complete desmosomes are internalized but that instead endocytosis clears the IgG induced aggregates of IgG, Dsg1 and PG from the membrane.

Further research should address the question how this endocytosis is related to acantholysis. We observed that endocytosis clears Dsg1 from the cells and that blistering takes place at the level where Dsg3 is no longer expressed. This suggests that acantholytic cells in PF might not be able to form desmosomes anymore as both Dsg1 and Dsg3 are lacking.

Acknowledgments

This work was in part supported by the Groningen Bernoulli Fund and a Schlumberger Foundation Faculty for the Future grant to DAMO.

References

1 Amagai. Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *J. Clin. Invest* 1994; **94**: 9.

2 Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67**: 869-77.

3 Stanley JR, Koulu L, Klaus-Kovtun V *et al.* A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 1986; **136**: 1227-30.

4 Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *J Clin Invest* 1984; **74**: 313-20.

5 Amagai M. Pemphigus as a paradigm of autoimmunity and cell adhesion. *Keio J Med* 2002; **51**: 133-9.

6 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J Clin Invest* 1999; **103**: 461-8.

7 Amagai M, Karpati S, Prussick R *et al.* Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic. *J Clin Invest* 1992; **90**: 919-26.

8 Berkowitz P, Hu P, Liu Z *et al.* Desmosome signaling. Inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization. *J Biol Chem* 2005; **280**: 23778-84.

9 Muller EJ, Williamson L, Kolly C *et al.* Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *J Invest Dermatol* 2008; **128**: 501-16.

10 Nguyen VT, Arredondo J, Chernyavsky AI *et al.* Pemphigus vulgaris acantholysis ameliorated by cholinergic agonists. *Arch Dermatol* 2004; **140**: 327-34.

11 Williamson L, Hunziker T, Suter MM *et al.* Nuclear c-Myc: a molecular marker for early stage pemphigus vulgaris. *J Invest Dermatol* 2007; **127**: 1549-55.

12 Aoyama Y., Nagai M, Kitajima Y.. Binding of pemphigus vulgaris IgG to antigens in desmosome core domains excludes immune complexes rather than directly splitting desmosomes. *Br J Dermatol.*

13 Mao X, Choi EJ, Payne AS. Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies. *J Invest Dermatol* 2009; **129**: 908-18.

14 Sato M, Aoyama Y, Kitajima Y. Assembly pathway of desmoglein 3 to desmosomes and its perturbation by pemphigus vulgaris-IgG in cultured keratinocytes, as revealed by time-lapsed labeling immunoelectron microscopy. *Lab Invest* 2000; **80**: 1583-92.

15 Calkins CC, Setzer SV, Jennings JM *et al.* Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. *J Biol Chem* 2006; **281**: 7623-34.

16 Delva E, Jennings JM, Calkins CC *et al.* Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism. *J Biol Chem* 2008; **283**: 18303-13.

17 Cirillo N, Gombos F, Lanza A. Changes in desmoglein 1 expression and subcellular localization in cultured keratinocytes subjected to anti-desmoglein 1 pemphigus autoimmunity. *J Cell Physiol* 2007; **210**: 411-6.

18 Wilgram GF, Caufield JB, Lever WF. [Electron Microscopic Studies in Skin Diseases with Acantholysis (Pemphigus Vulgaris, Pemphigus Familiaris Benignus Chronicus, Darier's Disease).]. *Dermatol Wochenschr* 1964; **147**: 281-92.

19 Iwatsuki K, Takigawa M, Imaizumi S *et al.* In vivo binding site of pemphigus vulgaris antibodies and their fate during acantholysis. *J Am Acad Dermatol* 1989; **20**: 578-82.

20 Iwatsuki K, Han GW, Fukuti R *et al.* Internalization of constitutive desmogleins with the subsequent induction of desmoglein 2 in pemphigus lesions. *Br J Dermatol* 1999; **140**: 35-43.

21 Tada J, Hashimoto K. Curvicircular intracytoplasmic membranous structures in keratinocytes of pemphigus foliaceus. *J Cutan Pathol* 1996; **23**: 511-7.

22 Vodegel RM, Jonkman MF, Pas HH *et al.* U-serrated immunodeposition pattern differentiates type VII collagen targeting bullous diseases from other subepidermal bullous autoimmune diseases. *Br J Dermatol* 2004; **151**: 112-8.

23 Wilgram GF, Caulfield JB, Lever WF. An electron microscopic study of acantholysis in pemphigus vulgaris. *J Invest Dermatol* 1961; **36**: 373-82.

24 Wilgram GF, Caulfield JB, Madgic EB. An Electron Microscopic Study of Acantholysis and Dyskeratosis in Pemphigus Foliaceus: With a Special Note on Peculiar Intracytoplasmic Bodies. *J Invest Dermatol* 1964; **43**: 287-99.

25 Berkowitz P, Diaz LA, Hall RP *et al.* Induction of p38MAPK and HSP27 phosphorylation in pemphigus patient skin. *J Invest Dermatol* 2008; **128**: 738-40.

26 Jolly PS, Berkowitz P, Bektas M *et al.* p38MAPK signaling and desmoglein-3 internalization are linked events in pemphigus acantholysis. *J Biol Chem*; **285**: 8936-41.

27 Berkowitz P, Hu P, Warren S *et al.* p38MAPK inhibition prevents disease in pemphigus vulgaris mice. *Proc Natl Acad Sci US A* 2006; **103**: 12855-60.

28 Berkowitz P, Chua M, Liu Z *et al.* Autoantibodies in the autoimmune disease pemphigus foliaceus induce blistering via p38 mitogen-activated protein kinase-dependent signaling in the skin. *Am J Pathol* 2008; **173**: 1628-36.

Chapter 6

Staphylococcal scalded skin syndrome: loss of desmoglein 1 in patient skin

A.S. Aalfs, D.A.M. Oktarina, G.F.H. Diercks, M.F. Jonkman and H.H. Pas

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

Published in *European Journal of Dermatology* 2010, 20(4):451-6

Abstract

Staphylococcal scalded skin syndrome (SSSS) is a blistering disease of the skin caused by an infection with certain strains of *Staphylococcus aureus. In vitro* studies have suggested that exfoliative toxins secreted by these bacteria cleave the desmosomal adhesion molecule desmoglein 1 leading to loss of cell-cell contact in the superficial epidermis. In this study we have investigated the fate of desmoglein 1 in biopsies of patients with SSSS to see whether the ectodomain of desmoglein 1 is cleaved. Our data largely confirm previous *in vitro* data. The different biopsies demonstrated loss of the ectodomain of desmoglein 1 to different degrees. The endodomain of desmoglein 1 meanwhile remained present. Most remarkably, in one of our patients the immunofluorescent analysis demonstrated that not desmoglein 1 but desmocollin 1, another desmosomal cadherin, became affected. This raises the question if other toxins and/or other bacteria than *Staphylococcus aureus* might also induce SSSS.

Introduction

Staphylococcal scalded skin syndrome is a skin disease that is characterized by generalized superficial flaccid blisters and denudation or desquamation [1]. The underlying cause of SSSS is a *Staphylococcus aureus* infection which produces exfoliative toxins (ETs) [1-4]. At first, patients present with fever, erythema and tenderness of the skin, later followed by widespread formation of fluid filled blisters that are thin walled and easily rupture, especially in regions of friction. Gentle friction on healed or uninvolved skin produces separation of keratinocytes of the superficial epidermis, a blister will form within minutes; this is called a positive Nikolsky sign, a characteristic phenomenon in SSSS [5]. Within hours to days large sheets of epidermis peel off, due to the loss of the roof of the blisters, which resembles scalded skin [1]. Denuded, sensitive and painful skin is left. Mucous membranes are never affected [1,5].

SSSS is a relatively uncommon disease that is mostly seen in newborns and young children, but may also occur in immune compromised patients or adults with renal failure [1-3]. The mortality rate in children is low, with early diagnosis and appropriate therapy, but still 3%. Potentially fatal complications include dehydration, hypothermia and the risk of secondary infections [5,6]. Long-term complications, such as scarring, are hardly ever seen because of the superficial level of the blister and the rapid healing after treatment [5]. In adults the mortality rate can reach over 50% despite aggressive antibiotic management, because the majority of this group suffers from an underlying disease or an immune compromised state [6,7].

In the 18th and 19th centuries SSSS in infants was called 'pemphigus neonatorum' because of the clinical similarities between SSSS and pemphigus; an autoimmune disease that also causes blistering of the skin [4]. There are two different subtypes of pemphigus, pemphigus foliaceus (PF) and pemphigus vulgaris (PV). In the case of PF blisters develop in the superficial layers of the epidermis, below the stratum corneum, with an identical histopathology as seen in SSSS [4,8]. The loss of keratinocyte adhesion in pemphigus is caused by immunoglobulin G (IgG) autoantibodies. The target molecules for these IgG autoantibodies in PF are recognized to be desmoglein (Dsg) 1 [1,4,9].

Desmogleins are proteins found in desmosomes; intercellular adhesion structures that interconnect the epidermal keratinocytes by anchoring the intermediate filaments of one cell to another. Dsg1 and -3 mediate this intercellular adhesion. Desmogleins are cadherins, a family of calcium dependent adhesion molecules, of which the members Dsg1 and Dsg3 and the desmocollins (Dsc) 1, 2 and 3 are expressed in skin. The cadherins demonstrate a specific distribution within the epidermal layers; Dsg1 and Dsc1 are more intensely present in the superficial layers, whereas Dsg3 and Dsc3 are more restricted to the lower layers [10].

In 1970 exfoliative toxins (ETs) were for the first time suggested to be involved in the pathogenesis of SSSS. Melish and Glasgow isolated *S. aureus* from SSSS patients and injected it into newborn mice. This resulted in intra-epidermal cleavage and exfoliation, resembling the manifestation in human disease [11].

Three isoforms of ET, exfoliative toxins A (ETA), B (ETB) and D (ETD) have been identified as being capable of inducing human SSSS [12,13,14]. In Europe and the USA the ma-

jority of cases involve ETA producing strains, while in Japan the ETB strains dominate [1,5,7,15]. These ETs are glutamic acid-specific serine proteases and in 2000 Amagai *et al.* demonstrated that Dsg1 is the substrate, the same desmosomal protein to which the IgG in PF is directed, that is specifically cleaved by ETA [8]. Two years later it was found that ETB had the same specificity [7]. Hanakawa *et al.* showed that human and mouse Dsg1 are cleaved by ETA and ETB at the same site, at glutamic acid 381, between extracellular domain (EC) 3 and EC 4 [2,16,17]. ETs also cause bullous impetigo, the localized form of SSSS. Here *S. aureus* gets through the skin barrier and releases the toxin locally causing blisters at the site of infection [6,8]. ET producing *S. aureus* can be isolated from the lesions in contrast to the situation in SSSS. In SSSS the toxins are produced at a distant focus and get into the circulation causing blisters at remote sites. Therefore *S. aureus* cannot be obtained from intact blisters. The diagnosis of SSSS is therefore verified by isolation of ETA and/or ETB producing *S. aureus* from other sites [17]. Commonly these include the conjunctivae, umbilicus, nasopharynx or blood [1,2,4,7].

Proof that ETs cause the lesions in SSSS and bullous impetigo through cleavage of Dsg1 is overwhelming but nonetheless only obtained through experimental studies that used S. *aureus* extracts or purified toxins ETA and ETB in mouse model systems or tissue sections of normal human skin. No study has addressed the fate of Dsg1 in patients in order to confirm the current hypothesis of pathogenesis. The aim of the present study therefore is to verify that Dsg1 is indeed cleaved by ETs *in vivo* in patient skin.

Material and methods

Patients

For this study we selected eight biopsies of eight different patients with staphylococcal scalded skin syndrome. All patients, except for one, were children at the time of diagnosis, ranging from an eight-day old neonate to the age of 7 years. The only exception was an 82 year old woman. The diagnose SSSS was based on clinical findings and histological aspects in the haematoxylin-eosin (HE) staining. Histological aspects included subcorneal blister formation, the degree of acantholysis and the presence of a minor infiltrate. Biopsies of healthy skin obtained from breast reduction were used as controls.

Biopsies

Punch biopsies were taken from lesional skin of patients with SSSS. Three biopsies had been frozen immediately after collection in liquid nitrogen and then been stored at -80°C. The other five biopsies were formalin-fixed, paraffin-embedded and stored at room temperature.

Immunofluorescence microscopy

From the frozen biopsies, cryostat sections with a thickness of 4 μm were cut and then mounted on Polysine™ glass slides. The slides were 15 minutes cold air-dried before a fan

before the staining procedure. From the formalin-fixed, paraffin embedded tissue blocks also 4 µm sections were cut and brought on Starfrost® glass slides. Sections were deparaffinised by Xylol followed by microwave treatment for antibody retrieval. Deparaffinised slides were placed in Tris HCl buffer (0.1 mol/L, pH 9.5) and placed in a 98°C microwave for 10 minutes. This procedure was followed three times, after which sections were ready for staining.

The slides were washed with phosphate-buffered saline (PBS pH 7.2) followed by incubation with the primary antibody (mouse monoclonals) diluted in PBS containing 1% (w/v) ovalbumin (PBS/OVA). Incubation time was 30 minutes in a moist chamber at room temperature. The sections were then washed with PBS for 15 minutes and, as a secondary step, incubated with Alexa488-conjugated goat anti-mouse IgG (Molecular Probes Eugene, OR, U.S.A) diluted in PBS/OVA 1% (dilution 1:600) for 30 minutes. For double staining with two different mouse monoclonals we used Zenon® Mouse IgG Labeling Kits Alexa Fluor®488 and Alexa Fluor®568 (Molecular Probes, Invitrogen, USA) by following technical protocols from the company. Nuclear counterstaining was performed with bisbenzimide diluted in PBS (8 µg/ml) for five minutes. After a final five minutes PBS wash the sections were coverslipped under SlowFade® antifade reagent (Molecular Probes, Invitrogen, USA). Slides were examined with a Leica DMRA fluorescence microscope at 40 times magnification. The staining patterns were photographed using a Leica DFC 350FX digital camera (Leica Microsystems AG, Wetzlar, Germany).

Antibodies

The ectodomain of Dsg1 was stained with Dsg1-P23 (dilution 1:20), Dsg3 with Dsg3-G194 (dilution 1:40), Dsc1 with U100 (1:40) and Dsc3 with U114 (all from Progen Immuno-diagnostika, Heidelberg, Germany). The endodomain of Dsg1 was stained with DG3.10 (1:10) (Acris Antibodies, Herford, Germany). Clone DG3.10 also recognizes Dsg2. Dsg2 mRNA is present in low amounts in epidermis and other stratified epithelia, however protein expression in normal human skin is that low that it is not detectable by immunofluorescence and therefore DG3.10 staining represents only Dsg1 [18,19]. Plakoglobin was stained with 5F11 (1:1000) (Sigma-Aldrich, Missouri, USA). All antibodies, except Dsg3-G194, reacted on paraffin embedded sections after microwave treatment.

Results

Immunofluorescence on normal human skin shows Dsg1 to be unevenly expressed throughout the epidermis. Expression starts at low level in the basal cells and then gradually increases towards the upper layers. When the uppermost layers are reached, expression fades again. When staining the patient biopsies for the ectodomain of Dsg1 we observed various staining patterns that we classified in four groups (Table 1). These were: (1) staining as in normal healthy skin, (2) a staining pattern that was unevenly distributed around the cells, (3) loss of staining when nearing a blister and (4) complete loss of intraepidermal staining throughout the biopsy although sometimes small intracellular dots remained visible which likely repre-

sent newly synthesized Dsg1 at the endoplasmatic reticulum (Fig. 1).

Biopsy #	Normal staining pattern	Uneven cellular distribution of Dsg1	Loss of Dsg1 towards the blister	Complete loss of Dsg1
1				+
2		+		
3		+	+	
4		+	+	
5		+		
6	+			
7			+	
8				+

Table 1. Staining patterns of the Dsg1 ectodomain in patient specimens. Biopsies 1-5 are paraffin-embedded skin biopsies. Biopsies 6-8 are frozen skin biopsies.

The disappearance of Dsg1 staining fits in with the current hypothesis that Dsg1 is degraded by *S.aureus* ET. We used an ectodomain binding monoclonal, and the Dsg1 ectodomain is attacked and spliced off at position Glu381 by ET. To investigate if loss of the ectodomain also results in loss of the complete protein we also stained our sections for the endodomain. The endomain remained present in the sections, also at positions where the staining of the ectodomain was lost (Fig. 2). Near the blister the staining however could appear distorted, becoming more cytoplasmic, possibly as a secondary effect to blistering.

In addition we also investigated our biopsies for aberrant expression of other cadherins –Dsg3, Dsc1 and Dsc3 – and for the desmosomal plaque protein plakoglobin. In all SSSS biopsies Dsc3, Dsg3, and plakoglobin kept their normal distribution similar to that in normal human skin (data not shown). Dsg3 could only be investigated in frozen biopsies as this monoclonal was not suitable for staining paraffin sections. Dsc1 also remained present in most biopsies but in the greater part of biopsy #6 surprisingly the membrane staining of Dsc1 was lost and replaced by a diffuse cytoplasmic staining. At the same time the Dsg1 molecule was present with a normal distribution (Fig. 3).

Discussion

In this study we investigated whether the ectodomain of Dsg1 is cleaved in the skin of patients with SSSS. In all biopsies but one the normal staining pattern of the ectodomain of Dsg1 was not preserved. In contrast the staining pattern of the Dsg1 endodomain remained normal in the skin. Similar observations were done by Amagai *et al.* when searching the target of ETs [7,8]. They incubated normal human skin sections with ETA and ETB, and demonstrated loss of cell surface Dsg1 but not of the cytoplasmic domain of Dsg1 [7]. Total loss of the Dsg1 throughout the whole biopsy, as in the *in vitro* experiments of Amagai, we saw only in

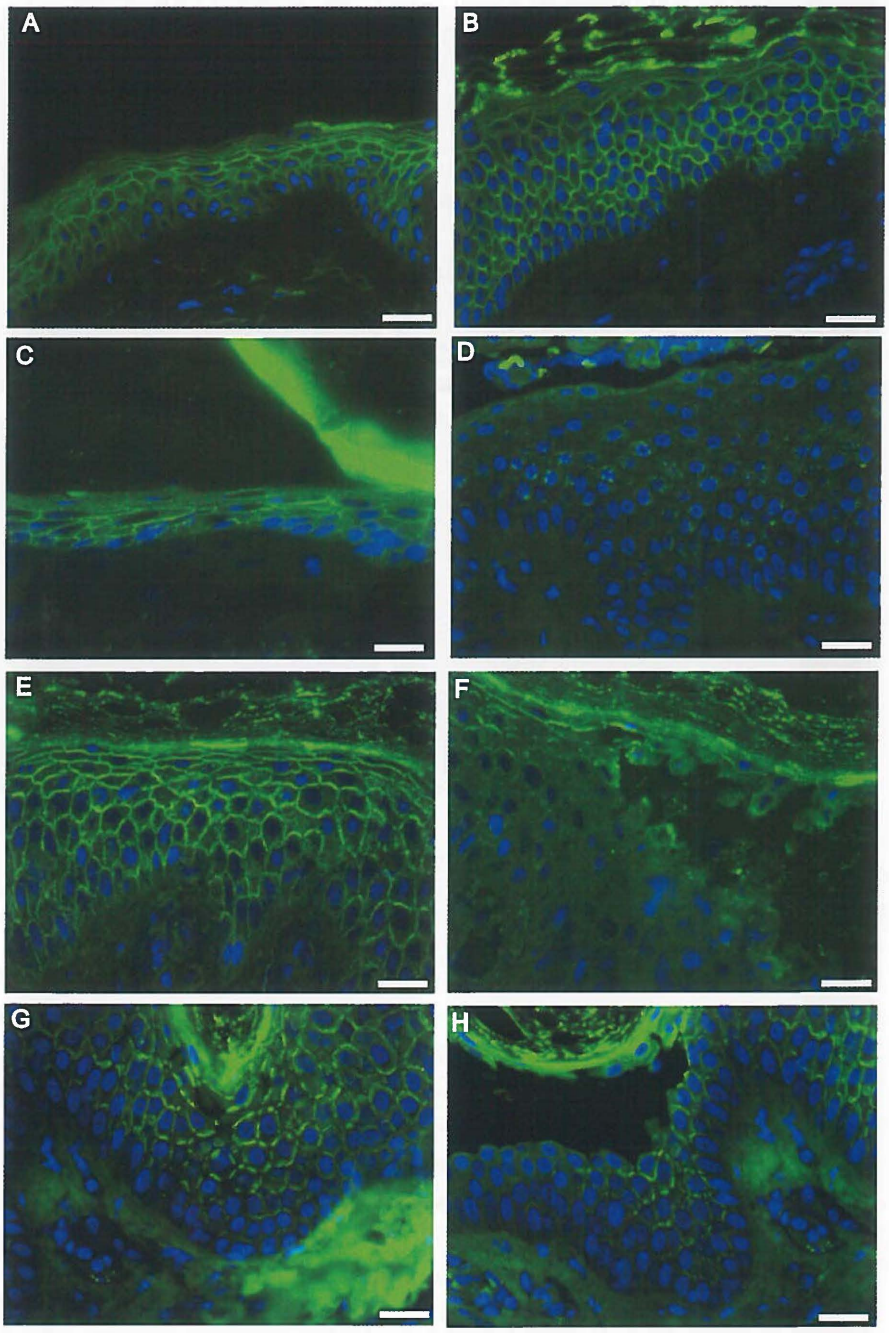


Figure 1. Immunofluorescence of the Dsg1 ectodomain in normal and SSSS patient skin. The ectodomain of Dsg1 in normal human skin (**A**, frozen section and **B**, paraffin section) has a diamond-shaped distribution with most expression in the midlayers fading out to the upper and lower layers. In patient skin the staining ranged between normal (**C**, biopsy #6) to completely absent (**D**, biopsy #1). In other biopsies Dsg1 could be normally present at one site (**E**) but completely lost near the blister (**F**) (biopsy #7). Also uneven distribution of Dsg1 along the cell membrane was seen (**G**) with loss at the site of the blister (**H**) (biopsy#3). Nuclei are in blue. White bar is 25 micrometers.

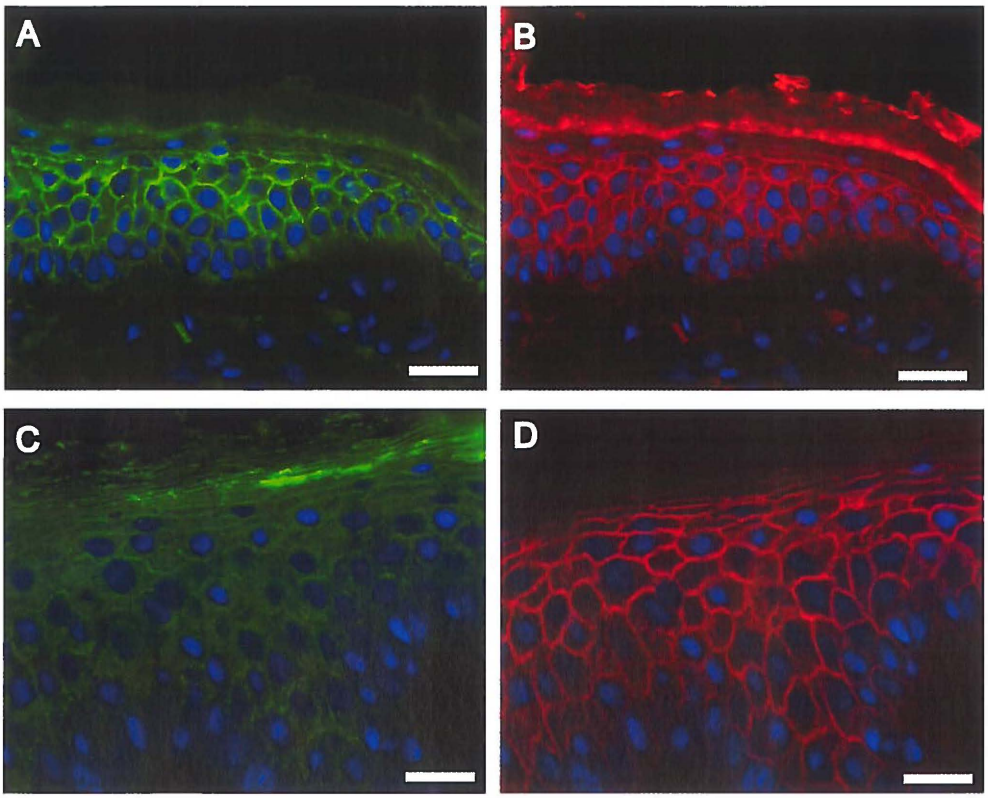


Figure 2. The endomain of Dsg1 remains present in patient skin. Skin sections were double stained for the Dsg1 ectodomain (green) and endodomain (red). In normal human skin both domains are evenly expressed along the cell membrane (**A**,**B**). In SSSS the endomain is still present as the ectodomain has been lost (**C**,**D**). Nuclei are in blue. White bar is 25 micrometers.

two of our biopsies. In three of our eight biopsies Dsg1 was only lost at sites near the blister. Four out of the eight biopsies showed a pattern where Dsg1 partially disappeared, with an uneven tissue distribution. The smooth pattern along the cell membrane no longer exists, but instead smaller and bigger gaps without Dsg1 are present in this pattern. In two biopsies both uneven cellular distribution as well as loss of Dsg1 near the blister was seen. Apparently the toxins do not diffuse evenly into the epidermis and Dsg1 is lost at those positions where sufficient active ET is present. *In vitro* studies have demonstrated that cleavage of Dsg1 by ETs is both a time- and dose-dependent process [7,8]. Thus, both toxin load and duration of infection will likely affect the degree of Dsg1 loss as observed in our patient biopsies.

The biopsy of one of our patients demonstrated affected Dsc1 instead of Dsg1. This particular patient was an 82 year old woman and also the only adult patient included in this study. She was hospitalized because of both heart failure and mild renal failure. During her hospitalization she developed blisters and epidermolysis that were diagnosed as SSSS. Because of her renal impairment she was already at risk of developing SSSS. Skin cultures were taken and subsequently treatment was started with flucloxacillin. As a result the lesions

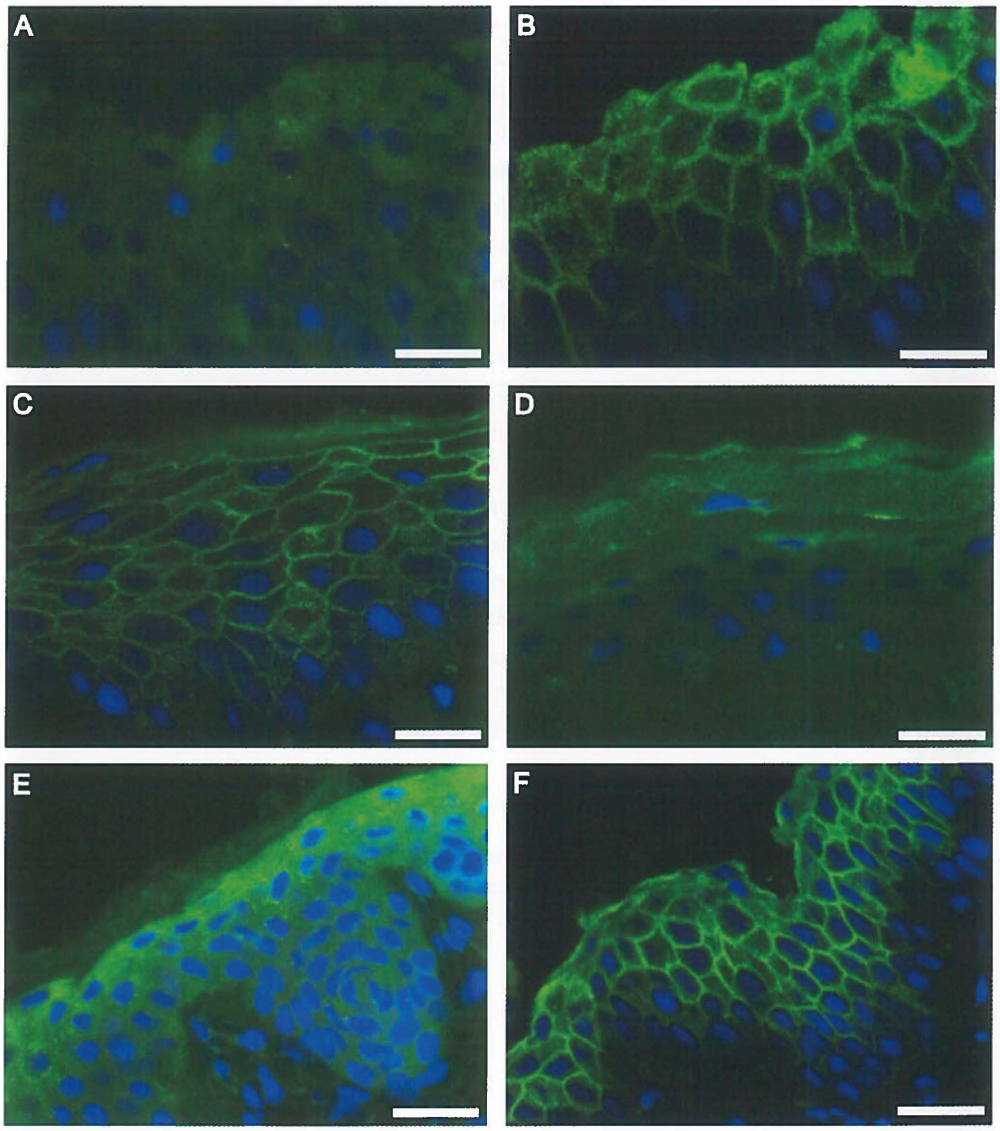


Figure 3. Loss of desmocollin 1 in biopsy #6. Alternate SSSS biopsy sections were stained in parallel for Dsg1 (**A,C,E**) and Dsc1 (**B,D,F**) and images were taken at the same positions of the epidermis. In the biopsy of patient #6 (**C,D**) Dsg1 remained present while Dsc1 vanished. In contrast in the biopsies of patients #7 (**A,B**) and #8 (**E,F**) Dsc1 remained normally present even if Dsg1 was lost. Nuclei are in blue. White bar is 25 micrometers.

healed which clinically confirmed the diagnosis. The cultures however did not demonstrate *S. aureus* and no expression of ETA and/or ETB genes could be demonstrated by PCR. Instead the skin cultures and one of the blood cultures turned out to contain a coagulase negative staphylococcus (CNS). Coagulase negative staphylococci are ordinary commensals of the skin [20]. They are non-virulent and are mainly considered contaminants when found in blood or other cultured specimens. A few strains of CNS's isolated from skin lesions have

been demonstrated to produce superantigenic exotoxins[21]. But to our knowledge it has never been reported that these toxins can produce skin infections like SSSS or bullous impetigo. Interestingly very recently a single case of a *Staphylococcus sciuri* scalded skin syndrome was reported[22]. S. *sciuri* constitutes for 0.79 to 4.3% of the CNS's and although they are associated principally with animals, they may also colonize humans. In humans infection with S. *sciuri* is most frequently associated with wound infections[23]. For our case, it can not be ruled out that CNS toxins were responsible for the disappearance of Dsc1. As at that time no investigation was performed to identify toxins, we cannot retrieve any information which might support possible toxin activity. The fact that no *S. aureus* was isolated from the cultures also does not by definition exclude its presence. If so, it remains however unexplained that Dsc1 and not Dsg1 became affected. No studies to the possible involvement of Dsc1 in SSSS exist, although Amagai *et al.* did investigate if desmocollin was affected by S. *aureus* ETs but found no evidence for this[7]. We also demonstrated that Dsg3, Dsc3 and plakoglobin were not affected by the loss of the Dsg1 ectodomain or the ETs themselves. Therefore desmosomal adhesion seems preserved in the lower layers as predicted by the desmoglein compensation hypothesis[24]. Blisters will thus appear when Dsg3 is no longer expressed, i.e. in the subcorneal layers.

In conclusion: where previous studies demonstrated that ETs could induce the histology of SSSS in neonatal mouse skin or normal human skin, in this study we confirmed that the immunofluorescence pattern of Dsg1 *in vivo* appeared gone or partially gone in SSSS patient skin. In addition we found loss of Dsc1 instead of Dsg1 in an adult patient.

Acknowledgments

This work was in part supported by the Groningen Bernoulli Fund and a Schlumberger Foundation Faculty for the Future grant to DAMO.

References

1 Nishifuji K, Sugai M, Amagai M. Staphylococcal exfoliative toxins: 'Molecular scissors' of bacteria that attack the cutaneous defense barrier in mammals. *J Dermatol Sci* 2007: 49: 21-31.

2 Hanakawa Y, Stanley JR. Mechanisms of blister formation by Staphylococcal toxins. *J Biochem* 2004: 136:747-750.

3 Anzai H, Stanley JR, Amagai M. Production of low titers of anti-desmoglein 1 IgG autoantibodies in some patients with staphylococcal scalded skin syndrome. *J Invest Dermatol* 2006: 126:2139-2141.

4 Stanley JR, Amagai M. Pemphigus, bullous impetigo, and the staphylococcal scalded skin syndrome. *N Engl J Med* 2006: 355:1800-1810.

5 Ladhani S. Recent developments in staphylococcal scalded skin syndrome. *Clin Microbiol Infect* 2001: 7:301-307.

6 Ladhani S. Understanding the mechanism of action of the exfoliative toxins of Staphylococcus aureus. *FEMS Immunol Med Microbiol* 2003: 39:181-189.

7 Amagai M, Yamaguchi T, Hanakawa Y, Nishifuji K, Sugai M, Stanley JR. Staphylococcal exfoliative toxin B specifically cleaves desmoglein 1. *J Invest Dermatol* 2002: 118:845-850.

8 Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR. Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nat Med* 2000: 6:1275-1277.

9 Payne AS, Hanakawa Y, Amagai M, Stanley JR. Desmosomes and disease: pemphigus and bullous impetigo. *Curr Opin Cell Biol* 2004: 16:536-543.

10 Green KJ, Simpson CL. Desmosomes: new perspectives on a classic. *J Invest Dermatol* 2007: 127:2499-2515.

11 Melish ME, Glasgow LA. The staphylococcal scalded skin syndrome: development of an experimental model. *N Engl J Med* 1970: 282:1114-1119.

12 Kondo I, Sakurai S, Sarai Y. New type of exfoliatin obtained from staphylococcal strains belonging to phage groups other than group II, isolated from patients with impetigo and Ritter's disease. *Infect Immun* 1974: 10:851-861.

13 Ladhani S, Joannou CL, Lochrie DP, Evans RW, Poston SM. Clinical, microbial and biochemical aspects of the exfoliative toxins causing staphylococcal scalded skin syndrome. *Clin Microbiol Rev* 1999: 12:224-242.

14 Yamaguchi T, Nishifuji K, Sasaki M, Fudaba Y, Aepfelbacher M, Takata T, Ohara M, Komatsuzawa H, Amagai M, Sugai M. Identification of the Staphylococcus aureus etd pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun* 2002: 70:5835-5845.

15 Patel GK, Finlay AY. Staphylococcal scalded skin syndrome: diagnosis and management. *Am J Clin Dermatol* 2003: 4:165-175.

16 Hanakawa Y., Schechter NM, Lin C, *et al.* Molecular mechanisms of blister formation in bullous impetigo and staphylococcal scalded skin syndrome. *J Clin Invest* 2002: 110:53-60.

17 Whittock NV, Bower C. Targetting of desmoglein 1 in inherited and acquired skin diseases. *Clin Exp Dermatol* 2003: 28:410-415.

18 Schäfer S, Koch PJ, Franke WW. Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins. *Exp Cell Res* 1994: 211:391-399.

19 Iwatsuki K, Han GW, Fukuti R, Ohtsuka M, Kikuchi S, Akiba H, Kaneko F. Internalization of constitutive desmogleins with the subsequent induction of desmoglein 2 in pemphigus lesions. *Br J Dermatol* 1999: 140:35-43.

20 Roth RR, James WD. Microbial ecology of the skin. *Annu Rev Microbiol* 1988: 42:441-464.

21 Akiyama H, Yamasaki O, Tada J, Arata J. The production of superantigenic exotoxins by coagulase-negative staphylococci isolated from human skin lesions. *J Dermatol Sci* 2000: 24:142-145.

22 Tang Y. A case report of Staphylococcus sciuri scalded skin syndrome. *Chinese Journal of Contemporary Pediatrics* 2008: 10:264.

23 Stepanović S, Dakić I, Morrison D, *et al.* Identification and characterization of clinical isolates of members of the Staphylococcus sciuri group. *J Clin Microbiol* 2005: 43:956-958.

24 Mahoney MG, Wang Z, Rothenberger K, Koch PJ, Amagai M, Stanley JR. Explanation for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J Clin Invest* 1999: 103:461-468.

Chapter 7

General discussion and future perspectives

D.A.M. Oktarina

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

Recent studies on pemphigus pathogenesis

The understanding of the pathogenesis of pemphigus has developed considerably in the last few decades. In 1964 Beutner and Jordon began the modern history of pemphigus by discovering the circulating autoantibodies against keratinocytes cell surface in PV [1]. These autoantibodies were then shown to be pathogenic, as they could induce blistering in skin organ cultures [2,3] and by passive transfer study in neonatal mice [4]. The target antigens of PF and PV were then later identified using immunoprecipitation and immunoblot to be 160- and 130-kDa glycoproteins that were later identified as desmogleins 1 and 3, respectively [5-8]. The basic pathology of pemphigus, regarding the clinical phenotype and the localization of blister, is explained by what is now known as the desmoglein compensation theory [9-11]. Nevertheless, the molecular mechanism of blister formation in pemphigus remains a subject of debate.

The modern pemphigus story began from the steric hindrance hypothesis, explaining that the binding of IgG to desmoglein may simply block and disrupt homophilic as well as heterotypic trans-interaction and then lead to the splitting of desmosomes and loss of cell-cell adhesion [9]. However, keratinocytes of PG-deficient mice did not respond to pemphigus IgG what suggested that intracellular molecules were involved in the pathomechanism [12]. The cell signalling hypothesis was then proposed as an alternative explanation that takes into account the intracellular molecules, most significantly PG. This hypothesis suggests that the antibody-antigen interaction activates intracellular signalling events leading to the acantholysis. Another important observation was that PV IgG could deplete Dsg3 from cells, probably by endocytosis of IgG bound Dsg3 [13]. This led to a third alternative hypothesis, that of disturbed desmosomal assembly through desmoglein depletion [13-15].

Choice to study pemphigus pathogenesis in patient skin

We deliberately choose patient biopsies to study pemphigus pathogenesis. Most research is done trough experimental model systems, often cultured cells and sometimes mouse models. None of these models is perfect. *In vitro* models show a low ability of autoantibodies to block cell cohesion in cell culture, in contrast to the high efficacy *in vivo* (commentary of Robert Gniadecki in [25]). Furthermore their desmosomal composition is different from skin cells. Also animal models have been used to investigate the role of the immune system in PV, such as using passive transfer of patient IgG to the neonatal mice, however these mice lack the immune competent response (commentary of Ahmed and Fidkis-Hareli in [25]). Mouse models have given immense understanding into the disease mechanism, however mouse is not completely equivalent to human, thus some questions remain that will have to be addressed in human models. The human *in vitro* models have their advantages and disadvantages (for review see [26]). Hence no single model may be preferred, but various models can be used in a complementary manner. In patient skin we can study the actual pathogenesis of the disease in *the scene of the crime*. The Centre for Blistering Diseases, Department of Dermatology University Medical Centre Groningen is the perfect location for such studies as many patients with blistering diseases all over the Netherlands and sometimes also from abroad are referred

to here. Thereby they have a large collection of patient biopsies that, after being used for diagnosis, were stored at -80°C. This method enables us to trace back what happened in patient skin and draw conclusions about the most likely events that took place in the patient skin. Studying a human disease using actual patient skin approaches the *in vivo* human environment as close as possible, however this method also has its limitation. What we see in patient skin is a frozen state of the pathogenesis, and we cannot modify any internal factors. For this reason, it is also more difficult to follow the time course of the disease development. Human skin contains also a huge numbers of internal factors and situations that we cannot control and specify independently. Using patient tissue sample only for research is also ethically more difficult than using engineered organ or cell culture models.

A model of the pathogenesis of pemphigus

In **chapter 2**, we observed the granular IgG deposition pattern in skin of pemphigus patients. We also showed selective aggregation of the targeted Dsg induced by IgG. This aggregation of Dsg is specific for pemphigus as in **chapter 6** we demonstrate that in staphylococcus scalded skin syndrome, that gives identical blisters as PF, no aggregation of Dsg is seen. Using *in vitro* experiments we explained this selective aggregation as an effect of the cross-linking of Dsg due to the bivalency of the IgG. Similar to the Dsg3 depletion on cultured keratinocytes, we observed that disorganization and depletion took place in the patient skin. How do our data comply with the three main hypotheses on acantholysis? In my thesis, I have not studied acantholysis at the ultrastructural level by electron microscopy and therefore can not provide information on the actual splitting of the desmosome. However as we clearly show that IgG causes the targeted Dsg to become sequestered outside the desmosome we feel that steric hindrance is less likely. Our data also question the cell-signalling hypothesis. The cell-signalling hypothesis is largely based on cultured cell experiments with anti-Dsg3 patient serum. If binding of IgG to Dsg3 would activate pathways that lead to acantholysis then basal skin cells that have the highest epidermal expression of Dsg3 would be expected to demonstrate this acantholysis. This is not what is seen in patient skin. The cells are normal without any indication of cell-cell widening. That the IgG is active in skin and binds Dsg3 is clear from the colocalization of IgG and Dsg3 and the altered distribution of Dsg3. If binding of IgG to Dsg3 would lead to activation of pathways, in particular the p38MAPK pathway then skin cells apparently contain some sort of compensation mechanism. Our data best fit the third hypothesis, the Dsg3-depleted desmosome model, as in our biopsies the targeted Dsg concentrated in dots and did not colocalize with the other desmosomal cadherins anymore. In the hypothesis it is assumed that this leads to perturbed assembly of desmosomes. The detailed views how Dsg depletion affects desmosomes marginally differs between the supporters of the hypothesis. All agree that non-desmosmal Dsg3 is quickly removed from the membrane, later followed by desmosomal Dsg3. Calkins *et al.* and Aoyama *et al.* ascribe much importance to the removal of IgG bound desmosomal Dsg3 and subsequent internalization what they see as the crucial step of desmosomal loss. Mao *et al.* in contrast believe

that the removal of non-desmosomal Dsg3 is more essential as this will prevent desmosomal assembly. We favour the latter view. It should be realized that desmosomes themselves are very dynamic structures with high protein turnover. Cadherins are continuously displaced by newly synthesized molecules as has been demonstrated for PG, Dsg2 and Dsc2 in cultured cells [16,17]. Dsg3 therefore will be continuously removed and replaced in desmosomes and the additional supposed endocytosis of IgG bound desmosomal Dsg3 will not make much difference here. Our data on Dsg1 demonstrated that a similar depletion mechanism is happening in PF skin but now for Dsg1 instead of Dsg3. We therefore favour the following hypothesis. Desmosomes are dynamic structures that are continuously renewed, partly by the formation of completely new desmosomes, partly by replacement of molecules in existing desmosomes [16,17]. Newly synthesized cadherins are transported from the Golgi to the membrane and form floating puncta. Plakoglobin will bind and when puncta fuse to larger puncta other desmosomal proteins will bind and finally keratin intermediate filaments will attach [18]. These newly formed structures then are able to incorporate into existing desmosomes or will fuse together to a new desmosome. In pemphigus IgG will bind all structures that contain Dsg but it are the small floating Dsg rafts without attached filaments that will be removed by endocytosis [18]. This removal of newly formed Dsg prevents it from being incorporated into desmosomes. In skin this, in case of anti-Dsg3 antibodies, will lead to Dsg3-depleted desmosomes and in case of anti-Dsg1 antibodies to Dsg1-depleted desmosomes. Yet, both types of depleted desmosomes apparently still can fulfil their adhesive task and will keep the cells attached to each other. This binding is still that strong that Nikolsky's sign cannot be provoked as we have shown for skin of PV oris patients, while in PF the blister forms in the higher layers of the skin, where Dsg3 is not expressed. In Nikolsky negative PF skin we expect the Dsg1 depletion to be incomplete, the remaining Dsg1 is sufficient to maintain adequate desmosomal strength and thus the threshold of impaired assembly has not yet been reached. If further loss of Dsg1 occurs then desmosomes cannot be renewed anymore and will melt away due to normal turnover, leaving cells that have lost adhesive properties and become acantholytic (Figures 1 and 2).

In **chapter 3** we studied whether the depletion took place in the mucosal tissue of mucosal dominant PV (solely anti-Dsg3) and PF (anti-Dsg1) patients. As in pemphigus skin, *in vivo* bound IgG manifests in mucosa in a partly granular pattern. In the PV mucosa the same Dsg3 clusters were found as in skin. Above the blister other aggregates were present that, as they contained all desmosomal components, we interpreted as clustered desmosomes probably secondary to the blistering underneath. We were interested in the PF mucosa as we had found widening between the cells in the basal and suprabasal layers of PF skin. Moreover in a morphological study of endemic PF patients Guedes *et al.* had found widening in the lower layers of oral mucosa, such as foreskin, uterine cervix and vagina wall [19]. Indeed widening seems present in the PF mucosa. In skin widening correlated with clustering of Dsg1 and PG, and when we stained the PF mucosa we observed that in the lower two layers granular depositions of IgG were present that contained PG and Dsg1. The finding of Dsg1 clusters in

the lower layers contradicts the current concept of Dsg1 distribution in the oral mucosa. Our results suggest another distribution than currently found in articles and textbooks. Instead of expression in the highest mucosal layers we reverse this concept and suggest that Dsg1 is expressed in the lower two layers. The compensation hypothesis suggests that the blistering in mucosa is due to the absence of Dsg1. The question is then raised whether Dsg1 compensates for Dsg3 loss in mucosal PV in the basal and suprabasal layer. In two of the three mucosal PV biopsies we investigated we indeed found a higher split level. The lowest two cell-layers are beneath the blister, in favour of Dsg1 compensating for Dsg3 loss. Our concept predicts that the row of tombstones will only form in PV with additional anti-Dsg1 antibodies. Our data are based on only few biopsies but they make clear that a deep investigation of cadherin distribution in mucosal tissue is needed. Furthermore future studies should also concentrate on the role of Dsg1 in the desmosome. Our studies have shown that Dsg3 loss does not have a great impact on the cells, while Dsg1 depletion leads to non-acantholytic intercellular widening. In **chapter 2** we already shortly discussed the relation between widening and Dsg1. The crucial question seems to be if the widening is caused by the depletion of Dsg1 from the desmosome or by the heavy clustering of Dsg1 together with plakoglobin. The first studies to be done should be investigating defined depleted skin by electron microscopy to study the depleted Dsg1-desmosomes and depleted Dsg3-desmosomes.

Basement membrane deposition in pemphigus erythematosus

Dsg1 also is central to pemphigus erythematosus (PE), also known as Senear-Usher syndrome. In PE the skin contains IgG depositions along the basement membrane zone (BMZ), also known as a 'lupus band', preferably at sun-exposed sites [20,21]. In **chapter 4** we have demonstrated that this 'lupus-band' consists of IgG, complement C3c and the ectodomain of Dsg1. Based on the fact that our patients had received UV-therapy we hypothesized that PE is a photosensitive form of PF, and placed PE next to CDLE and SCLE as photosensitive autoimmune diseases. Our hypothesis is deducted from patient skin biopsies. Future research should test our hypothesis. First experiments to do would include UV-irradiation of sun-unexposed skin of PE and PF patients after which the skin should be investigated for BMZ deposition. Furthermore we do not know how UV-light induces the shedding of the Dsg1 ectodomain. As discussed UV-radiation might stimulate the release of specific chemokines that recruit inflammatory cells leading to keratinocyte damage and proteolytic loss of the Dsg1 ectodomain [22]. Such concept deserves further attention.

Endocytosis of desmoglein

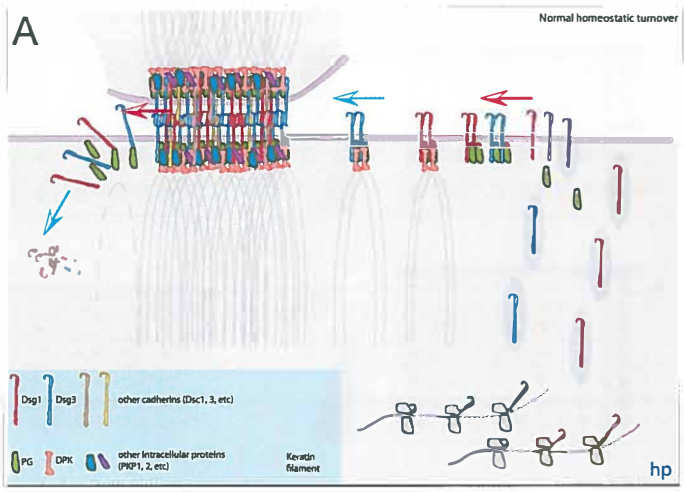
There is no doubt that in cultured keratinocytes PV IgG induces the internalization of Dsg3 molecules by endocytosis. It has been known for a decade that the primary effect of the PV IgG binding is the internalization of the non desmosomal Dsg3 [18]. A few years later, studies showed that the internalization of IgG-Dsg3 complex is through a clathrin- and dynamin-independent pathway[14]. In 2007 Cirillo also demonstrated the internalization of Dsg1 by PF

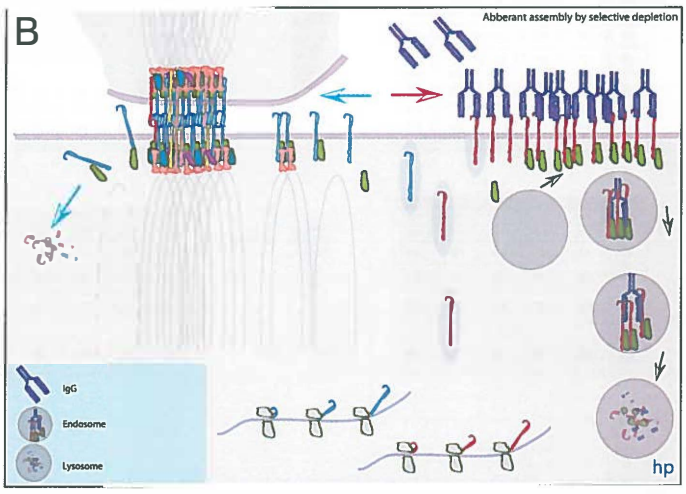
Figure 1. (Opposite page) Proposed model of pemphigus acantholysis.

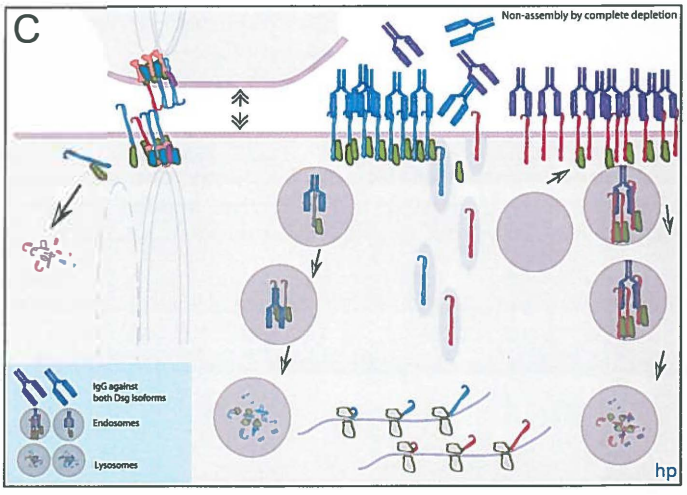
A. Under normal conditions desmosomal proteins are continuously renewed. Different desmogleins (red and light blue) are synthesized and transported to the membrane. Plakoglobin (green) attaches and small patches form. Then these patches develop further and keratin filaments attach, implying that at least desmoplakin (pink) must have bound to the plakoglobin. These latter patches can now fuse into an existing desmosome. In which phase other desmosomal proteins as desmocollins and plakophilins (other rainbow colors) enter the desmosome is presently unknown. Proteins are in continuous turnover in the desmosome and older proteins will be discarded off by cellular uptake and will be destroyed or recycled (fragments).

B. When patients have IgG specific for only one desmoglein than this IgG will bind only one desmoglein isoform, and the soluble desmoglein in the membrane will be crosslinked by the IgG and gather in clusters. This way the flow of freshly synthesized desmogleins towards the desmosome is interrupted what leads to selective desmoglein depleted desmosomes (**Chapter 2**). This does not lead to acantholysis as the not-targeted desmoglein isoform can compensate for the depletion. The clusters of crosslinked Dsg are removed by endocytosis (gray ballons) and finally destroyed (fragments) in lysosomes (**Chapter 5**).

C. When IgG is present to both desmoglein isoforms then desmosomes will become depleted of both desmoglein 1 and 3. When both desmogleins are absent desmosomes are not stable. Existing desmosomes are still subject to the same protein turnover as normal and will therefore, by lack of fresh replacement molecules, melt away. Without desmosomes cells do not have sufficient adhesive properties and cell separation, or acantholysis, will occur.


A
Normal homeostatic turnover
Dsg1
Dsg3
other cadherins (Dsc1, 3, etc)
PG
DPK
other intracellular proteins (PKP1, 2, etc)
Keratin filament
hp


B
Abberant assembly by selective depletion
IgG
Endosome
Lysosome
hp


C
Non-assembly by complete depletion
IgG against both Dsg isoforms
Endosomes
Lysosomes
hp

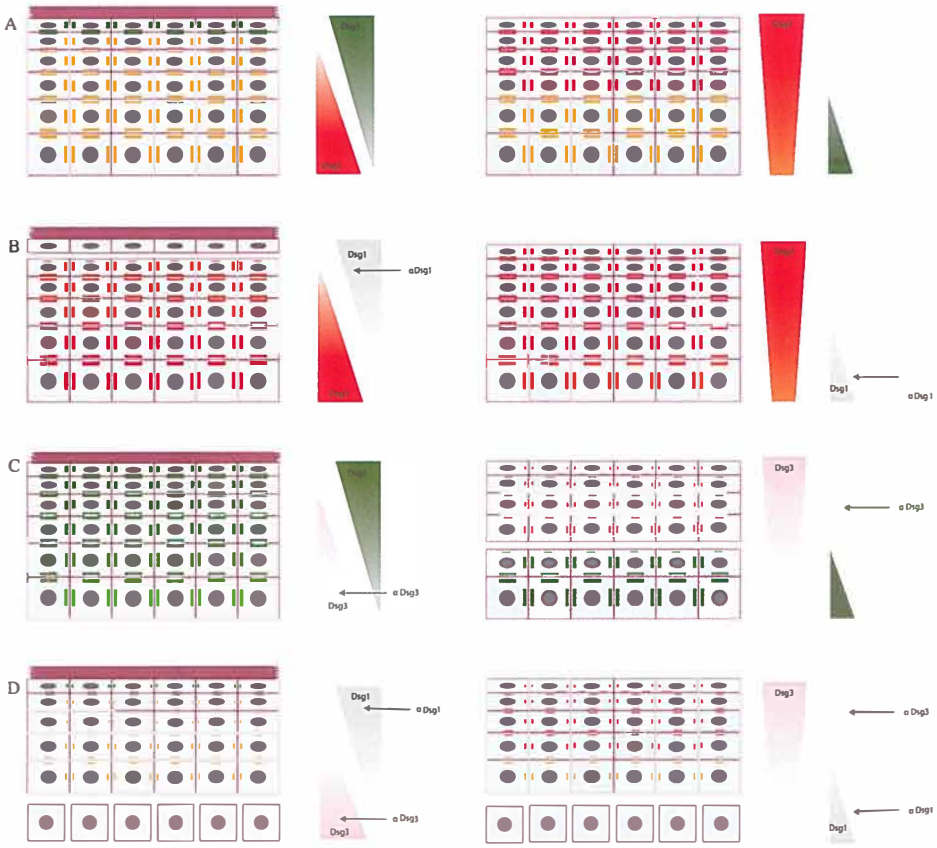

A
B
C
D
Dsg1
Dsg3
α Dsg1
α Dsg3

Figure 2. (Opposite page) Blister level and desmoglein isoform distribution.

A. When Dsg1 (green) and Dsg3 (red) are expressed in the skin and mucosa, desmosomes contain both desmogleins (yellow desmosomes) except subcorneal layer where they contain only Dsg1 (green). In the mucosa higher layers only contain Dsg3 (red desmosomes).

B. When anti-Dsg1 autoantibodies are present, the blister forms superficially. The lower layer of epidermis contains Dsg1-depleted desmosomes that still function through compensation of Dsg3 (red desmosomes), while the superficial layer looses its desmosomes as Dsg3 is not expressed here. In mucosa (right) all desmosomes contain Dsg3 (red desmosomes), thus no blister is formed.

C. When anti-Dsg3 antibodies are present the blisters form in the mucosa. The epidermis contains Dsg3-depleted desmosomes that still function through compensation of Dsg1 (green desmosomes) throughout all layers and no blister is formed. In mucosa (right), desmosomes become Dsg3-depleted, but in the two lower layers Dsg1-compensation prevents acantholysis (green desmosomes) and the blister forms just above were Dsg1 is not expressed.

D. When anti-Dsg1 and anti-Dsg3 autoantibodies are present, the blister is formed suprabasally in both the epidermis and the mucosa. Due to depletion of Dsg1 and Dsg3, desmosomes do not form anymore. In the layers above depletion does take place but there is still sufficient demosomal strength to keep the cells together (represented by smaller desmosomes). The split occurs at suprabasal level as the basal cells are the first that contact the antibodies. Cell-separation occurs between the basal and the suprabasal layer as the basal cells remain attached to dermal compartment. As all desmosomes disappear basal cells demonstrate the tombstone phenomenon.

IgG, which started with the early depletion of Dsg1 from the adhesion complex[23]. We have investigated our patient biopsies for endocytosis of Dsg1 and Dsg3. Surprisingly we could not find proof of endocytosis of Dsg3 in PV skin or mucosa. Although early endosomes were present we were unable to detect Dsg3 in these. It can be that the amount of Dsg3 taken up is low and that our techniques are not sensitive enough to detect the internalized Dsg3. Endocytosis of Dsg1 however we could clearly detect. In **chapter 5** we demonstrate endocytosis of IgG, Dsg1 and PG in PF skin, and we concluded that the endosomes clear the IgG/Dsg1/PG aggregates from the cell membrane. Our conclusion thereby questions previous electron microscopic work that suggested that complete desmosomes were taken up. What surprised us was that the endocytosis that we observed was only present in higher cell layers. Although endosomes are present in lower layers they do not take up Dsg1 here. The reason for this is at present unclear. Perhaps the pathway used for endocytosis is only present in differentiated cells. Future research should therefore characterize this pathway. Endocytosis was present in lesional skin underneath the blister what suggests that endocytosis is connected with blistering. The question is if this really is the case. In our model we hypothesise that desmosomes become depleted of Dsg1 what leads to desmosomal loss in the superficial layer that does not synthesize the compensating Dsg3. This model explains acantholysis without a role for endocytosis. Endocytosis would then only clear the aggregated proteins from the membrane but not contribute to the actual pathomechanism. On the other hand we do not know much about the clusters. We observed that endocytosis of a cluster is often symmetrically by two adjacent cells, what implies that the cluster of one cell spatially localizes with the cluster of a neighbouring cell. Perhaps these clusters are connected by IgG and do provide some adhesion. In such case, although unlikely, endocytosis would have pathogenic meaning. There is no doubt that further research into these clusters is warranted, preferably at the ultrastructural level through immunoelectron microscopy. Another item to be investigated is the role of p38MAPK inhibitors in PF pathology. Berkowitz *et al.* demonstrated in a mouse model that inhibiting p38MAPK prevents PF blistering, what made them suppose that cell-signalling is involved in acantholysis [24]. These findings clash with our simple depletion model. The p38MAPK however is a central signalling molecule. We do not know if it is also involved in regulating desmosomal dynamics. If so, another mode of action could be inhibiting desmosomal renewal and interrupt depletion. The *in vitro* skin model we described in **chapter 2** would be an attractive model for further investigating the role of the p38MAPK inhibitors.

References

1 Beutner EH, Jordon RE. Demonstration of Skin Antibodies in Sera of Pemphigus Vulgaris Patients by Indirect Immunofluorescent Staining. *Proc Soc Exp Biol Med* 1964; **117**: 505-10.

2 Schiltz JR, Michel B. Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. *The Journal of investigative dermatology* 1976; **67**: 254-60.

3 Farb RM, Dykes R, Lazarus GS. Anti-epidermal-cell-surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteinase. *Proceedings of the National Academy of Sciences of the United States of America* 1978; **75**: 459-63.

4 Anhalt GJ, Labib RS, Voorhees JJ *et al.* Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. *The New England journal of medicine* 1982; **306**: 1189-96.

5 Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *The Journal of clinical investigation* 1984; **74**: 313-20.

6 Stanley JR, Yaar M, Hawley-Nelson P *et al.* Pemphigus antibodies identify a cell surface glycoprotein synthesized by human and mouse keratinocytes. *The Journal of clinical investigation* 1982; **70**: 281-8.

7 Eyre RW, Stanley JR. Human autoantibodies against a desmosomal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients. *J Exp Med* 1987; **165**: 1719-24.

8 Hashimoto T, Ogawa MM, Konohana A *et al.* Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. *The Journal of investigative dermatology* 1990; **94**: 327-31.

9 Amagai M. Autoimmunity against desmosomal cadherins in pemphigus. *Journal of dermatological science* 1999; **20**: 92-102.

10 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *The Journal of clinical investigation* 1999; **103**: 461-8.

11 Udey MC, Stanley JR. Pemphigus--diseases of antidesmosomal autoimmunity. *JAMA* 1999; **282**: 572-6.

12 Caldelari R, de Bruin A, Baumann D *et al.* A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris. *The Journal of cell biology* 2001; **153**: 823-34.

13 Aoyama Y, Kitajima Y. Pemphigus vulgaris-IgG causes a rapid depletion of desmoglein 3 (Dsg3) from the Triton X-100 soluble pools, leading to the formation of Dsg3-depleted desmosomes in a human squamous carcinoma cell line, DJM-1 cells. *The Journal of investigative dermatology* 1999; **112**: 67-71.

14 Calkins CC, Setzer SV, Jennings JM *et al.* Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. *The Journal of biological chemistry* 2006; **281**: 7623-34.

15 Mao X, Choi EJ, Payne AS. Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies. *The Journal of investigative dermatology* 2009; **129**: 908-18.

16 Windoffer R, Borchert-Stuhltrager M, Leube RE. Desmosomes: interconnected calcium-dependent structures of remarkable stability with significant integral membrane protein turnover. *Journal of cell science* 2002; **115**: 1717-32.

17 Gloushankova NA, Wakatsuki T, Troyanovsky RB *et al.* Continual assembly of desmosomes within stable intercellular contacts of epithelial A-431 cells. *Cell and tissue research* 2003; **314**: 399-410.

18 Sato M, Aoyama Y, Kitajima Y. Assembly pathway of desmoglein 3 to desmosomes and its perturbation by pemphigus vulgaris-IgG in cultured keratinocytes, as revealed by time-lapsed labeling immunoelectron microscopy. *Laboratory investigation; a journal of technical methods and pathology* 2000; **80**: 1583-92.

19 Guedes AC, Rotta O, Leite HV *et al.* Ultrastructural aspects of mucosas in endemic pemphigus foliaceus. *Archives of dermatology* 2002; **138**: 949-54.

20 Chorzelski T, Jablonska S, Blaszczyk M. Immunopathological investigations in the Senear-Usher syndrome (coexistence of pemphigus and lupus erythematosus). *The British journal of dermatology* 1968; **80**: 211-7.

21 Giannetti A. Immunofluorescence studies in the Senear-Usher syndrome. *Arch Dermatol Forsch* 1974; **248**: 287-96.

22 Lehmann P, Homey B. Clinic and pathophysiology of photosensitivity in lupus erythematosus. *Autoimmunity reviews* 2009; **8**: 456-61.

23 Cirillo N, Gombos F, Lanza A. Changes in desmoglein 1 expression and subcellular localization in cultured keratinocytes subjected to anti-desmoglein 1 pemphigus autoimmunity. *J Cell Physiol* 2007; **210**: 411-6.

24 Berkowitz P, Chua M, Liu Z *et al.* Autoantibodies in the autoimmune disease pemphigus foliaceus induce blistering via p38 mitogen-activated protein kinase-dependent signaling in the skin. *The American journal of pathology* 2008; **173**: 1628-36.

25 Amagai M, Ahmed AR, Kitajima Y *et al.* Are desmoglein autoantibodies essential for the immunopathogenesis of pemphigus vulgaris, or just "witnesses of disease"? *Experimental dermatology* 2006; **15**: 815-31

26 van der Wier G, Pas HH, Jonkman MF. Experimental human cell and tissue models of pemphigus. *Dermatol Res Pract*; **2010**: 143871.

Summary

D.A.M. Oktarina

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

Pemphigus pathogenesis

Pemphigus is an autoimmune disease of the skin and/or mucous membranes characterized by circulating IgG that deposits intraepidermally. The two major types of pemphigus comprise pemphigus vulgaris (PV), always affecting mucosa and in approximately half of the cases also skin, and pemphigus foliaceus (PF) that affects only skin. The IgG against desmoglein 3 (Dsg3) in PV is responsible for suprabasal acantholysis while IgG against Dsg1 causes subcorneal acantholysis in PF[1-4].

Desmogleins, members of the cadherin family, are desmosomal proteins that link keratinocytes. Two opposing desmogleins bind each other with the extracellular N-terminal domains. Their cytoplasmic domains are indirectly connected to the keratin filaments through desmoplakin (DP) that binds intermediate filaments, and plakoglobin (PG), also known as γ-catenin, that links desmoplakin to the cytoplasmic tails of the cadherins. Dsg3 is abundantly present in the lower epidermis but absent in the subcorneal layer, while Dsg1 expression increases from the basal to the subcorneal layer. This differential distribution is the basis of the desmoglein compensation hypothesis that explains the difference in separation levels between PV and PF by Dsg isoform redundancy [5,6].

The mechanism by which IgG induces acantholysis remains a subject of debate. Three main models prevail today. According to the first model, the binding of IgG to Dsg may disrupt homophilic transinteraction, leading to lengthwise splitting of desmosomes and loss of cell-cell adhesion [7]. The second model proposes that the binding of IgG to Dsg derails intracellular signaling pathways ending with cytoskeleton collapse or desmosome disassembly [8-12]. A hallmark observation here was that PG-deficient keratinocytes did not respond to pemphigus IgG, implicating the involvement of cytoplasmic proteins in the pathology [13]. The third model suggests that the IgG interferes with desmosome dynamics leading to severe assembly problems and loss of desmosomes [14]. For all three models supportive evidence is present in the literature underlining the vast complexity of investigating the pathogenic process of acantholysis. Most of today's concepts originate from experimental models and relatively little attention has been given to patient skin. People who perform diagnostic immunofluorescence (IF) microscopy know that there is a discrepancy between *in vivo* IgG deposition by direct IF on patient skin and the intercellular substance (ICS) pattern by indirect IF that is typical for pemphigus. The ICS pattern is a smooth staining around the epidermal cells, also called honeycomb or chicken wire pattern, and is in line with the normal distribution of the desmogleins. In pemphigus patient epidermis, however, IgG deposits in an uneven granular pattern not reflecting the normal desmoglein (Dsg) distribution [15].

In **chapter 2** we investigated multiple pemphigus patient skin biopsies in order to understand why the IgG deposits *in vivo* in such an aberrant granular fashion. We analyzed the expression pattern of desmosomal proteins in patient skin and demonstrated that the IgG deposition mimics reallocation of the pemphigus antigens. In pemphigus foliaceus (PF) Dsg1 loses its smooth membrane distribution, starting with the basal layer, and concentrates in clusters overlying the cell membrane. Similar clustering, but now of Dsg3, is present in pure mucosal pemphigus vulgaris (PV), while in skin of mucocutaneous PV patients with

antibodies to Dsg1 and Dsg3, both desmogleins aggregate. Also plakoglobin (PG) is affected and co-concentrates with IgG and Dsg. This is far more prominent in PF and mucocutaneous PV than in pure mucosal PV, indicating that disturbance of Dsg1 has much greater impact on PG than disturbance of Dsg3. The same shifts in distribution could be induced *in vitro* in normal human skin by pemphigus patient IgG, but not by Fab fragments of the same IgG. Nevertheless these Fab fragments induced acantholysis. In areas with heavy PG clustering widening between cells occurred. This study shows that IgG induced disorganization of Dsg and PG is not essential for acantholysis but is related to non-acantholytic cell-cell widening. In this study we conclude that the IgG induced rearrangement of the Dsg autoantigens is responsible for the granular IgG deposition pattern in patient skin. In PF and in mucocutaneous PV skin Dsg1 aggregation, but not Dsg3 aggregation, correlates with non-acantholytic intercellular widening. In this chapter we propose a model of pemphigus pathogenesis in which we favor the idea that Dsg3 becomes sequestered outside desmosomes by the polyvalent character of IgG and desmosomal assembly becomes perturbated.

In **chapter 3** we studied the IgG deposition patterns in the mucous membranes by correlating these to the distribution of desmosomal and non-desmosomal components. In mucosal PV in basal cells beneath the blister we found clusters with the same composition -IgG, Dsg3 and PG- as in healthy mucosal PV skin. Above the blister these clusters were also found but here also a second type of cluster was present that contained IgG and all desmosomal components. We conclude that these latter clusters must represent aggregated desmosomes. In non-blistering mucosa of a PF patient we found the same clusters as in the skin of PF patients. These clusters were present in the basal and suprabasal layer. Similar to PF skin the cells in these layers demonstrated intercellular widening. That aggregated Dsg1 is present in the lower layers of the mucosa questions current theory on cadherin distribution in oral mucosa.

In **chapter 4** we examined biopsies and sera of three patients with basement membrane zone (BMZ) IgG deposition and fourteen patients without BMZ IgG deposition. We then retrospectively analyzed patient files for clinical manifestations. In this study we showed that the BMZ deposits differed from the intraepidermal deposits. Plakoglobin was absent but Dsg1 was present although not as the full-length molecule but rather as a spliced-off part of the ectodomain. The Dsg1 containing deposits were located around the lamina densa. All three patients reacted weakly by ELISA to BP230, however no BP230 was found in the BMZ deposits. The typical PE butterfly facial rash was definitely found in two patients. ANA antibodies were absent in all patients. Remarkably all three patients were initially misdiagnosed as having psoriasis and had received light therapy prior to their visit to our clinic. We conclude that the presence of Dsg1 ectodomain fragments along the BMZ explains why the IgG deposits here. The spliced-off Dsg1 ectodomain indicates the presence of an additional to PF pathogenic mechanism. Clinically BMZ Dsg1 ectodomain deposition corresponds with PE.

Older immunoelectron microscopic studies have suggested that in PF skin endocy-

tosis of desmosomes takes place. In **chapter 5** we aimed to verify this endocytosis and, if present, to investigate its relation with the IgG aggregates. We performed double immunofluorescence staining on 10 skin biopsies of six PF patients for the following molecules: IgG, Dsg1 and 3, desmocollin 1 and 3, PG, desmoplakin, plakophilin 3, early endosomal marker 1 (EEA1) and cathepsin-D. EEA1 did not colocalize with the IgG aggregates in the basal and suprabasal layers but did contact them in the layers above. In these same cells endosomes were present in the cytoplasm. These endosomes contained IgG, Dsg1 and PG but not other cadherins or plaque components. Endocytosis was present in lesional skin but not in non-lesional skin. Our results confirm that endocytosis of IgG bound components takes place in skin of PF patients. Based on the molecular composition of the endosomal cargo we conclude that these endosomes do not take up complete desmosomes but instead clear the IgG/Dsg1/PG aggregates from the cell membrane.

Staphylococcal scalded skin syndrome (SSSS) is a blistering disease of the skin which shows generalized and superficially exfoliative lesions similar to PF. In SSSS patient skin we did not observe the clustering of Dsg1 as in PF, what demonstrated that the clustering is unique to pemphigus pathogenesis and not a secondary result of blistering. *In vitro* studies have suggested that the blistering in SSSS is due to Staphylococcus aureus exfoliative toxins that cleave the desmosomal adhesion molecule Dsg1 leading to loss of cell-cell contact in the superficial epidermis. In **chapter 6** we have investigated the fate of Dsg1 in biopsies of patients with SSSS to see whether the ectodomain of Dsg1 is cleaved. Our data largely confirm previous *in vitro* data. The different biopsies demonstrated loss of the ectodomain of Dsg1 to different degrees. The endodomain of Dsg1 meanwhile remained present. Most remarkably, in one of our patients the immunofluorescent analysis demonstrated that not Dsg1 but desmocollin 1, another desmosomal cadherin, became affected. This raises the question if other toxins and/or other bacteria than *Staphylococcus aureus* might also induce SSSS.

All studies were performed in the Center for Blistering Diseases of the University Medical Center Groningen, to where many patients with blistering diseases, both inherited and acquired, from all around the Netherlands and sometimes from other countries are referred. The diagnostic and laboratory facilities in this center enabled us to investigate the actual pathogenesis of various blistering diseases, especially pemphigus, from the *crime scene* of the disease. Our choice of studying patient skin was justified by the results. Apart from that our observations agreed primarily with one of the main hypotheses on acantholysis we also found new aspects of the disease that could not have been unveiled by cultured cell models for lack of proper desmosomal desmoglein composition. Our results indicate a far larger role for Dsg1 in the basal cell than currently assumed, what has great implications for the current views on the pathogenesis of pemphigus. This thesis therefore calls for further research into the role of Dsg1 in the desmosome and the role of anti-Dsg1 antibodies in the mechanism of acantholysis.

References

1 Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991; **67**: 869-77.

2 Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *The Journal of clinical investigation* 1984; **74**: 313-20.

3 Stanley JR, Koulu L, Klaus-Kovtun V *et al.* A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 1986; **136**: 1227-30.

4 Amagai M, Hashimoto T, Shimizu N *et al.* Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *The Journal of clinical investigation* 1994; **94**: 59-67.

5 Amagai M. Pemphigus as a paradigm of autoimmunity and cell adhesion. *The Keio journal of medicine* 2002; **51**: 133-9.

6 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *The Journal of clinical investigation* 1999; **103**: 461-8.

7 Amagai M, Karpati S, Prussick R *et al.* Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic. *The Journal of clinical investigation* 1992; **90**: 919-26.

8 Berkowitz P, Hu P, Liu Z *et al.* Desmosome signaling. Inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization. *The Journal of biological chemistry* 2005; **280**: 23778-84.

9 Nguyen VT, Arredondo J, Chernyavsky AI *et al.* Pemphigus vulgaris acantholysis ameliorated by cholinergic agonists. *Archives of dermatology* 2004; **140**: 327-34.

10 Delva E, Jennings JM, Calkins CC *et al.* Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism. *The Journal of biological chemistry* 2008; **283**: 18303-13.

11 Muller EJ, Williamson L, Kolly C *et al.* Outside-in signaling through integrins and cadherins: a central mechanism to control epidermal growth and differentiation? *The Journal of investigative dermatology* 2008; **128**: 501-16.

12 Williamson L, Hunziker T, Suter MM *et al.* Nuclear c-Myc: a molecular marker for early stage pemphigus vulgaris. *The Journal of investigative dermatology* 2007; **127**: 1549-55.

13 Caldelari R, de Bruin A, Baumann D *et al.* A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris. *The Journal of cell biology* 2001; **153**: 823-34.

14 Mao X, Choi EJ, Payne AS. Disruption of desmosome assembly by monovalent human pemphigus vulgaris monoclonal antibodies. *The Journal of investigative dermatology* 2009; **129**: 908-18.

15 Burge SM, Wilson CL, Dean D *et al.* An immunohistological study of desmosomal components in pemphigus. *The British journal of dermatology* 1993; **128**: 363-70.

Nederlandse samenvatting

D.A.M. Oktarina

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

Dit proefschrift beschrijft studies naar het pathomechanisme van acantholyse – dit is het verlies van adhesie tussen aangrenzende cellen – bij de ziekte pemphigus. In tegenstelling tot het merendeel van de huidige studies is gekozen om de huid van patiënten in plaats van een model systeem als vertrekpunt te nemen, aangezien in patiëntenhuid met zekerheid de 'werkelijke' pathogenese moet plaatsvinden.

Pemphigus is een auto-immuun gemedieerde blaarziekte van de huid en slijmvliezen welke gekenmerkt wordt door intraepidermale depositie van immuunglobuline G (IgG). De twee hoofdvormen van pemphigus zijn pemphigus vulgaris (PV) en pemphigus foliaceus (PF). Bij PV zijn altijd de slijmvliezen aangedaan en in ongeveer de helft van de gevallen ook de huid. Bij PF is alleen de huid aangedaan. Bij PV vindt de blaarvorming suprabasaal plaats terwijl bij PF de blaren zich subcorneaal vormen. De autoantilichamen zijn gericht tegen desmogleïnes, bij PV altijd tegen desmogleïne 3 (Dsg3) en in een aantal gevallen ook tegen desmogleïne 1 (Dsg1) terwijl bij PF uitsluitend antilichamen tegen Dsg1 worden gevormd.

Desmogleïnes maken deel uit van de cadherine eiwitfamilie en zijn transmembrane eiwitten van het desmosoom. Desmosomen zijn symmetrische structuren van 300-400 nm doorsnee welke de keratinefilamenten van een cel met de keratinefilamenten van een naburige cel verbinden. Twee tegenover elkaar gelegen desmogleïnes binden aan elkaar met hun extracellulaire N-terminale domeinen. De cytoplasmatische domeinen zijn dan weer verbonden met de keratine filamenten via plakoglobin (PG), ook bekend als γ-catenine, en desmoplakine. Dsg3 is met name aanwezig in de onderste lagen van epidermis, maar is afwezig in de subcorneale laag, terwijl omgekeerd de expressie van Dsg1 toeneemt van de basale laag naar de subcorneale laag. Dit verschil in distributie vormt de basis van de desmogleïne compensatie hypothese welke het verschil in blaarniveau tussen PV en PF verklaart vanuit deze verschillende expressiepatronen.

Het mechanisme waardoor IgG acantholyse induceert is onderwerp van veel discussie. Er zijn drie belangrijke hypotheses. Volgens de eerste hypothese, de 'steric hindrance hypothesis' zou de binding van het IgG aan de desmogleïnes de transinteractie van deze desmogleïnes verstoren ten gevolge van sterische hindering. Hierdoor zou het desmosoom overlangs splijten in twee half-desmosomen waardoor de cellen van elkaar loslaten. De tweede hypothese, de 'cell-signalling hypothesis' gaat er vanuit dat binding van IgG aan desmogleïne intracellulaire signaal transductie routes activeert wat dan zou leiden tot ineenstorting van het cytoskelet of ontmanteling van desmosomen. Een 'hallmark' observatie hier was dat PG-deficiënte keratinocyten niet reageerden op pemphigus IgG wat suggereerde dat cytoplasmatische eiwitten in de pathologie betrokken waren. De derde hypothese, de 'desmosomal depletion hypothesis' veronderstelt dat het IgG interfereert met de turnover dynamiek van het desmosoom hetgeen leidt tot verstoring van de opbouw van het desmosoom doordat desmogleïnes niet meer ingebouwd kunnen worden hetgeen uiteindelijk tot verlies van desmosomen leidt. Voor alle drie hypothesen kan ondersteunend bewijs gevonden worden in de literatuur, hetgeen aangeeft hoe complex het onderzoek naar de pathogenese van acantholyse is.

Het merendeel van de huidige concepten en ideeën zijn gebaseerd op waarnemingen

aan experimentele modellen en relatief weinig aandacht is besteed aan de gebeurtenissen in de huid van de patiënten zelf. Degenen die routinematig diagnostisch immunofluorescentie (IF) onderzoek aan de huid verrichten weten dat er een discrepantie bestaat tussen de in vivo IgG afzetting in de huid van de patiënten en de in vitro aankleuring van gezond epitheel-substraat door patiënten IgG in het zogenaamde typische pemphigus ICS (intercellulaire substantie) patroon. Het ICS-patroon is een glad aankleuringspatroon rond de keratinocyten, ook wel bekend als 'honingraat' of 'kippengaas' patroon, en is in overeenstemming met de normale verdeling van de desmogleïnes. In pemphigus patiëntenhuid epidermis hebben de in vivo IgG posities echter vaak een andere vorm waarbij het gladde patroon plaatsmaakt voor een meer geclusterd, granulair beeld, wat niet overeenstemt met de normale desmogleïne distributie.

In hoofdstuk 2 onderzochten we meerdere huidbiopten van pemphigus patiënten om te begrijpen waarom de IgG depositie in vivo in een dergelijk afwijkend granulair patroon laat zien. We analyseerden de expressiepatronen van desmosomale eiwitten in patiëntenhuid en lieten zien dat het IgG-patroon een herverdeling van de pemphigus antigenen volgt. In pemphigus foliaceus (PF) verliest Dsg1 het gelijkmatige membraan distributie patroon. Dit proces begint al in de basale laag, en het Dsg1 concentreert zich in clusters ter plekke van de celmembranen. Vergelijkbare clustering, maar nu van Dsg3, vindt plaats in huid van mucosale pemphigus vulgaris (PV) patiënten , terwijl in de huid van mucocutane PV patiënten, welke antistoffen tegen zowel Dsg1 als Dsg3 hebben, beide desmogleïnes zijn geaggregeerd tot clusters. Ook de plakoglobin (PG) distributie verandert en colocaliseert met het IgG en Dsg. Dit is veel prominenter in PF en mucocutane PV huid dan in mucosale PV huid, wat aangeeft dat reallocatie van Dsg1 een veel sterker effect heeft op PG dan de reallocatie van Dsg3. Dezelfde verschuivingen in distributie konden in vitro geïnduceerd worden in normale humane huid door pemphigus patiënten IgG, maar niet door Fab-fragmenten van hetzelfde IgG. Dit geeft aan dat het IgG verantwoordelijk is voor de verandering in het Dsg distributiepatroon. In gebieden met zware PG clustering zagen we verwijding tussen de cellen in gebieden waar geen acantholyse optreedt. Dit suggereert dat IgG geïnduceerde desorganisatie van Dsg en PG niet essentieel is voor acantholyse, maar wel gerelateerd is aan niet-acantholytische cel-cel verwijding. In dit hoofdstuk concluderen we dat de IgG geïnduceerde desorganisatie van de Dsg autoantigenen verantwoordelijk is voor het granulaire IgG depositie patroon in de patiënten huid. Verder concluderen we dat er een correlatie bestaat tussen niet-acantholytische intercellulaire verwijding en clustering van Dsg1, maar dat deze correlatie niet bestaat voor clustering van Dsg3 en intercellulaire verwijding. In dit hoofdstuk stellen we verder een model voor waarin we de pathogenese van de acantholyse verklaren door desmosomale depletie van het autoantigene desmogleïne. Dit model past bij de 'desmosomal depletion hypothesis'.

In hoofdstuk 3 hebben we de IgG-depositie patronen bestudeerd in de slijmvliezen van pemphigus patiënten. In lesionale mucosa van mucosale PV patiënten vonden we in de basale cellen onder de blaar clusters met dezelfde samenstelling van IgG, Dsg3 en PG als in

de gezonde huid van deze patiënten. Deze waren ook boven de blaar aanwezig maar hier vonden we ook een tweede type cluster dat IgG bevatte en alle desmosomale componenten. We concluderen dat deze laatste clusters hoogstwaarschijnlijk aggregaten van desmosomen zijn. In gezond slijmvlies van drie PF patiënten vonden wij dezelfde clusters als in de huid van PF patiënten. Deze clusters waren aanwezig in de basale en suprabasale lagen. Net als bij de PF huid was intercellulaire verwijding van de onderste lagen zichtbaar. Het is duidelijk dat hier verder onderzoek naar nodig is. Dat er Dsg1 aanwezig is in de onderste lagen van de mucosa plaatst vraagtekens bij de huidige ideeën over de cadherine distributies in het mondslijmvlies.

In hoofdstuk 4 onderzochten we biopten en sera van drie PF patiënten met additionele granulaire basaal membraan zone (BMZ) deposities van IgG en complement en veertien PF patiënten zonder dergelijke BMZ IgG deposities. Deze studie liet zien dat de samenstelling van de granulaire BMZ deposities anders is dan die van de granulaire intraepidermale deposities. Plakoglobine was afwezig, maar Dsg1 was aanwezig, hoewel niet als het 'full-length' molecuul, maar als een afgesplitst deel van het ectodomein. Deze granulaire deposities bevonden zich rond de lamina densa. Het serum van deze patiënten reageerde zwak in een BP230 ELISA assay, echter BP230 was niet aan te tonen in de BMZ deposities. De patiënten dossiers werden retrospectief geanalyseerd en twee patiënten bleken een vlindervormig erytheem in het gezicht te hebben zoals dat ook bij een subform van PF, pemphigus erythematosus, wordt gezien. ANA antistoffen, indicatief voor lupus erythematosus waarbij ook een vlindervormig exatheem aanwezig is, waren afwezig bij alle patiënten. Opmerkelijk was dat bij alle drie patiënten in eerste instantie een verkeerde diagnose, psoriasis, was gesteld ten gevolge waarvan ze lichttherapie hadden ontvangen voordat ze doorverwezen werden naar onze kliniek. In dit hoofdstuk concluderen we dat de aanwezigheid van Dsg1 ectodomein fragmenten langs de BMZ verklaart waarom de IgG deposities zich hier bevinden. Dat afgesplitst Dsg1 ectodomein aanwezig is langs de BMZ wijst op de aanwezigheid van additionele pathologie.In de literatuur is IgG depositie langs de BMZ eerder beschreven als typisch voor pemphigus erythematosus.

In vroegere immunoelectron microscopische studies is gesuggereerd dat in PF huid endocytose van desmosomen zou kunnen plaatsvinden. In hoofdstuk 5 hebben we onderzocht of er inderdaad endocytose plaatsvindt, en zo ja, wat dan de relatie van deze endocytose is met de IgG-aggregaten welke we in de voorgaande hoofdstukken hebben beschreven. Endosomen colocaliseerden niet met de IgG-aggregaten in de onderste lagen van de huid maar maakten wel contact met de aggregaten in de hogere lagen. In deze cellen waren ook in het cytoplasma endosomen aanwezig welke IgG, Dsg1 en PG bevattten maar geen van de overige cadherines of plaque componenten. Deze endocytose vindt plaats in lesionale huid. In dit hoofdstuk concluderen we dat er inderdaad endocytose van IgG-gebonden componenten plaatsvindt maar dat, gebaseerd op de moleculaire 'cargo'van deze endosomen, er geen sprake is van endocytose van hele desmosomen, maar dat deze endosomen de IgG/Dsg1/PG aggregaten opruimen.

Staphylococcal scalded skin syndrome (SSSS) is een blaarziekte van de huid met ge-

generaliseerde en oppervlakkige blaren welke histopathologisch niet te onderscheiden zijn van PF blaren. Ook bij SSSS lijkt Dsg1 betrokken te zijn bij de pathogenese. In vitro studies hebben gesuggereerd dat exfoliatieve toxines uitgescheiden door de bacterie Staphylococcus aureus de oorzaak zijn. Deze toxines kunnen specifiek Dsg1 knippen en dit zou leiden tot verlies van intercellulaire adhesie. In hoofdstuk 6 hebben we deze hypothese getoetst door Dsg1 te onderzoeken in biopten van SSSS patiënten. Inderdaad bleek in biopten van deze patiënten verlies van het Dsg1 ectodomein op te treden. Het endodomein bleef echter wel aanwezig. Onze data bevestigen dus de in vitro data namelijk een splitsing van het Dsg1 molecuul. Opmerkelijk was de bevinding dat bij een van de patiënten niet Dsg1 maar desmocolline 1, een ander cadherine, verdween. Dit roept de vraag op of ook andere toxines of bacteriën tot SSSS kunnen leiden. In de huid van SSSS patiënt werd geen clustering van Dsg1 gezien zoals bij PF; dus deze clustering is uniek voor pemphigus en geen secundair gevolg van blaarvorming.

Alle studies in dit proefschrift zijn uitgevoerd in het Centrum voor Blaarziekten van het Universitair Medisch Centrum Groningen. Veel patiënten met blaaraandoeningen, zowel erfelijk als verworven, uit geheel Nederland en soms uit andere landen worden hiernaar doorverwezen vanwege het hoge niveau van de diagnostiek en zorg. De diagnostiek- en researchfaciliteiten van het centrum stelden mij in staat om de pathogenese van pemphigus, op de "crime scene", namelijk de huid van patiënten, te onderzoeken. De keuze om huid te bestuderen werd gerechtvaardigd door de resultaten. Behalve dat onze waarnemingen overeen kwamen met een van de belangrijkste hypotheses over het acantholytisch mechanisme hebben we ook nieuwe aspecten van de pathogenese gevonden welke niet ontdekt hadden kunnen worden als we met een celkweek model hadden gewerkt. Onze resultaten wijzen op een veel grotere rol voor Dsg1 in de basale cellen dan dat momenteel aangenomen wordt, en dit heeft gevolgen voor de huidige concepten betreffende de pathogenese van pemphigus. Dit proefschrift maakt duidelijk dat verder onderzoek naar de rol van Dsg1 in het desmosoom en naar de rol van anti-Dsg1 antilichamen in het pathomechanisme van acantholyse dringend gewenst is.

Acknowledgments

D.A.M. Oktarina

Centre for Blistering Diseases, Department of Dermatology,
University Medical Centre Groningen, University of Groningen,
Groningen, the Netherlands

These four years have been a wonderful period of my life. I have met wonderful people in Groningen, learned new things and lived an exciting life in a quite different culture. It is my privilege to express here my sincere thanks to all people with whom I have worked together on this study and all people who contributed through their help and their friendship to the completion of this thesis.

First I would like to express my gratitude to my promotor, Professor Marcel Jonkman. One of the most important days for me was the day I met Professor Marcel Jonkman in the Dermatology Course held by the Dutch Foundation in the University of Indonesia in Jakarta. I was so fascinated by his talk on blister diseases and activities in the research team in his department. My first thought was "I want to do that, too!" Almost one year later, I got the most exciting e-mail ever: I was accepted as a PhD student in Groningen. Professor Marcel Jonkman, you are one of God's hands handed to me to make all these happen. Thank you for your enthusiasm, trust, encouragement, and constructive criticism during this study.

Together with Marcel Jonkman, my copromotor, Dr. Hendri Pas, made an enormous contribution to this study. Hendri, since the beginning of this project, you have given unlimited ideas, attention, and time. You have always welcomed me in your office to discuss not only the results I got the previous day, but also spontaneous ideas, and to express my confusion on some particular subject. You have taken care of almost everything related to my project. Beside the research, it has always been enjoyable to talk about cooking and gardening. Thank you for your endless patience, enormous support, motivation, and the strength you gave me to finish this study.

I would like to thank the members of the reading committee: Professor Hardyanto Soebono, Professor Arjan Vissink, and Professor Harry van Goor, for reading and judging my manuscript.

Furthermore, I feel privileged to express my thanks to Professor Hardyanto Soebono from Gadjah Mada University, Yogyakarta, Indonesia. Your support has been shown since I expressed my intention to do a PhD study abroad. Thank you for your continuous support and encouragement during this study.

Here I would also like to thank all the staff members of the Faculty of Medicine, especially of the Department of Dermatovenereology in Gadjah Mada University, Yogyakarta, and Direktorat Jenderal Pendidikan Tinggi, the Ministry of the National Education, for all their support during my study in Groningen.

My gratitude goes also to my colleagues from the Gadjah Mada University: Awalia Febriana, Punto Dewo, Margareta Rinastiti, Titik Nuryastuti, Rosa Amalia, and Yoyo Suhoyo, it was always nice to have you here in Groningen. Nana, Dita, Adit and Lukman, thank you for sharing with me the experience of being a student in the Netherlands. Lukman, we started together trying to get higher education abroad after we finished our medical school in Yogyakarta. You then taught me how to bike after we knew we would do our studies in the Netherlands. Thank you for all the patience and support. I wish you all the best with your PhD study in Germany. I thank also my friends who study in Australia and Japan: Bayu,

Bambang, Didik, and many others for the shared spirit to finish our studies.

I am honoured to have Gerda van der Wier and Ramon van Tol as my paranymphs. It has been a pleasure for me to work together with Gerda on pemphigus. We had a great time in our shared office and in all the conferences we visited together such as Kyoto, Otsu, and Bern with the pemphigus community. Gerda, I want to thank you also for your contribution to the first chapter of this thesis. Ramon, thank you for the cordial friendship during these years. It was always pleasant to discuss about Indonesia, cooking, gadgets and about your culinary adventures beautifully described in your food blog.

Together with Gerda, it has been fun to work in our office with Antoni. Your spontaneous character brought us joy at work and in congresses, especially in Japan.

I would like to take this opportunity to thank all staff members, researchers and AIOs at the department of dermatology in UMCG for their support and their nice company through the years. My gratitude goes to Professor P.J.Coenraads and Dr. Gilles Diercks for being the opponents in the defence ceremony. Gilles, I envy your spontaneous ideas and kindness; thank you for your collaboration in this study. I want to express my thanks to Dr. Marcellus de Jong for his ideas and useful discussions. My special thanks go also to Barbara Horváth, Marie-Louise Schuttelaar, and Sylvia Kardaun, for our discussions and their suggestions. Susanne, I thank you for your contribution to chapter 6 of this thesis. Jacqueline, it was always nice discussing with you and planning our promotions together. Marieke Bolling, thank you for the explanations and examples on how to arrange the promotion. Wingyan, Marjon, and Peter, we had a great time in Helsinki and it has always been fun talking with you. Annemarie, Jorrit, Annemieke, Berend, Noor, Laura, Nynke, Janine, Simone, Margriet, Anton, Wendi, Yan Yin, and Angelique, with your hospitality and fun you made every day in the department enjoyable.

This study would have not finished without the enormous help from the people at the Laboratorium Immunodermatology UMCG. Jannie and Gonnie, thank you both for teaching me how to cut the biopsies and do the staining. Guus, I am thankful for your help with the ELISA and Westernblot. Duco, it has been interesting working together; I appreciate your constructive criticism and informative input, not only in the research work but also in ordinary subjects such as discussing about Dutch humor. Thank you also for your help in preparing the defence. Miranda, thank you for always ordering the antibodies and many other things, reminding me the laboratory rules and for lending me your Russian language books for my vacation. Working in the laboratory with all of you was fun.

Even though I worked just for a short time in the electron microscopy department, I enjoyed all those moments doing the difficult experiments there. Han van der Want, Ben Giepmans, Bert Blaauw, Freark Dijk, Ruby Kalicharan and Jeroen Kuipers, I thank you all for the joyful moments full with your hospitality and helpfulness.

I feel very lucky to be the daughter of my lovely parents. Mama and papa, Endang Tri Setyowati and Mochammad Hariyadi, my prayer is always for you, who shower me with love, trust and support. You have been very understanding while I was doing this study. My thanks

go also to my lovely oma, Eyang Sulastri and my aunt, Endang Irawati, for the everyday prayers and encouragement. Other relatives that I cannot mention one by one thank you all for your support and prayers.

Studying and living abroad, far from home, for several years would have not been easy without the hospitality of the Jacobs and Jansen families. Oom Ralph and tante Milly are like parents for me, from whom I have also learned a lot about the Dutch language and culture. I appreciate so much your attention and love. Shirley and Belinda thank you for your friendship. Oom Rudy and tante Mien, thank you for your hospitality when I visited your beautiful home in Roden. You all have become new families for me.

My uncle and aunt in Hamburg, Bobby and Marlis Herman, and my cousin, Tatjana, you are such a lovely family. We shared beautiful moments and fun together. Hamburg has become my second favourite city after Groningen to spend weekends and holidays.

I thank also the family Tri Hartanto and Enik Meidiati with their lovely young boys, Hadian and Rayhan. We shared happiness as well as sorrow. We gave strength, encouragement and prayer to each other. We have become family and will always be.

I was quite lucky that I found my open-minded and golden-hearted best friends Shanti, Puri, Nani and Yunia who have then become like my own sisters. We have shared similar situations as students, facing the same happiness, difficulties, challenges and problems. I have always enjoyed the unforgettable weekend moments with these strong women. Shanti, although we have different characters, we also have many similarities. It was accidentally funny that the international office took the wrong folder for both of us because of our similar name. These four years were full of sharing moments, tears and laughs. I cannot thank you enough for all of these. I wish all the best for you all and hope that you will always be my sisters.

Together with Ike, Eko and Inne, Dini, Insanu, Intan and Guntur, Amel and Puti, Iging and Desti, Nandang and Nisa, Neng, Ari, Christina Avanti, Adhi, Titah, Wisnu's family, Astri, Yota, Adji, Teguh, Poppy, Robby, Iqbal, Lia Atwa, Rachma, Alia, Faizah, Erythrina, Astri, Nizar, Wahono, Christin, Okta, Kenzi, Seno Muhsin and many more Indonesian students in Groningen that I cannot possibly mention one by one, I really had a lot of fun in Groningen. This community is a big Indonesian family here in Groningen, which helps each one of us feeling at home.

I am so grateful having met Konstantinos and having shared time together. I appreciate your genuine kindness. I thank you also for your useful criticism and our discussions. My words are not enough to thank you for being always there for me, in good times and bad ones. Your hands were always there to help me with everything including the layout and the cover of this thesis. Not enough thanks again for your enormous patience, attention and help. My best wishes are for you, for the happiness and success in your life.

This study would have not been completed without the financial support of the Bernoulli Funds from the University of Groningen, the Graduate School for Drug Exploration (GUIDE), and the Faculty for the Future Program from the Schlumberger Foundation.

Most of all, Alhamdulillahi robbil 'alamien, all the praises and thanks be to Allah, who has designed my life so beautifully.